Identifying the larva of the fan mussel, *Atrina fragilis* (Pennant 1777) (Pinnidae)

Running title: *ATRINA FRAGILIS* LARVAE

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ABSTRACT

Measuring dispersal in rare sessile benthic species is important in the development of conservation measures such as MPA networks. However, efforts to understand dispersal dynamics for many species of conservation concern are hampered by a lack of fundamental life-history information. Here we present the first description of larvae of the fan mussel, *Atrina fragilis*, and examine key life-history traits that affect dispersal. Larval identification was accomplished using complementary molecular and morphologic techniques. *Atrina*-specific primers were designed by aligning *Atrina* COI sequences available in GenBank. As none of these were from UK specimens, primers were designed in the most conserved regions found across *A. fragilis* and its closest relative *A. chautardi*. A monthly time-series of zooplankton samples (2014–2015) suggests that *A. fragilis* follows the same pattern in spawning observed for other pinnids at temperate latitudes, with peak spawning in summer and winter. Average shell growth was estimated to be 6 µm d^{-1} based on presumed daily growth lines on larval shells. Measurements of the larval shell visible through the juvenile shell indicate a length of up to 770 µm at settlement. Using presumed daily growth lines, this translates into a pelagic larval duration of around 4 months.

INTRODUCTION

In benthic marine species with a sedentary adult life stage, the transport of planktonic larvae is central in determining the distribution, dynamics and viability of populations (Underwood & Fairweather, 1989; Sale *et al.*, 2005; Botsford *et al.*, 2009; D'Aloia *et al.*, 2013). However, for the majority of marine species larval transport remains poorly understood (Salinas-de-León, Jones & Bell, 2012) due to a lack of fundamental life-history information, such as the season of spawning, duration of the planktonic phase and behavioural traits of larvae (Brussard, 1991; Hendriks, van Duren & Herman, 2005). Moreover, for many rare species the inability to identify planktonic stages precludes the collection of such information.

In the present study we develop methods for identifying larvae of the fan mussel, *Atrina fragilis* (Pennant, 1777), and examine key life-history traits that affect dispersal. This is one of the largest and rarest bivalve molluscs occurring in northern European waters (Woodward, 1985) and the only member of the Pinnidae to inhabit UK waters. In the UK it is protected under law (UK Gov, 1981) and has been highlighted as one of the most threatened species requiring targeted conservation (JNCC, 2010). *Atrina fragilis* is sensitive to the effects of benthic fishing gears and its distribution is believed to have been impacted by the industrialization of fishing over the past half century (e.g. Fryganiotis, Antoniadou & Chintiroglou, 2013). The only known population in Scottish waters that may currently be described as an aggregation was discovered in 2009, during routine monitoring of a spoil-site in the Sound of Canna (SoC), which is an over-deepened (> 200 m) glacial trench with muddy and sandy sediments lying between the Isles of Rum and Canna in the Small Isles archipelago off the west coast of Scotland. The biology of *A. fragilis* is not well

understood, with information on its reproduction and early life-history particularly sparse. No descriptions of *A. fragilis* larvae exist in the literature.

Bivalve larvae are notoriously difficult to identify using classic morphological methods, particularly during their early stages (e.g. Garland & Zimmer, 2002; Hansen & Larsen, 2005). Nonetheless, various early taxonomists attempted systematic classification of bivalve larvae from their morphology (Bernard, 1895; Borisiak, 1909; Lebour, 1938; Rees, 1950; Yoshida, 1956; Miyazaki, 1962), while subsequent researchers produced keys with shape, dimensions, umbo character, colour, hinge-line length and hinge morphology to distinguish between species (Loosanoff, Davis & Chanley, 1966; Garland & Zimmer, 2002). Morphological identification is greatly facilitated by descriptions of sympatric bivalves that allow the comparison of morphological features and by knowledge of the bivalve fauna of an area (Garland & Zimmer, 2002; Rees, 1950). However, greater certainty in larval identification, particularly for the early stages where morphological identification is most challenging, is now possible through genetic approaches (Garland & Zimmer, 2002; Larsen *et al.*, 2005; Fonseca *et al.*, 2010). DNA barcoding, a molecular method that uses short species-specific DNA fragments within a particular gene to identify organisms, is an efficient, robust and standardized tool (Hajibabaei *et al.*, 2011). This technique requires an established reference library comprising DNA sequences from the same genomic region, so that the unknown query sequence can be identified and validated when compared with sequences generated from closely related species (Yoccoz, 2012).

Various authors have noted the large size and distinctive triangular shape of the larvae of pinnids (Bernard, 1895; Jørgensen, 1946; Rees, 1950; Ota, 1961; Malchus, 2004; Allen, 2011; Malchus & Sartori, 2013). Descriptions of varying detail

exist for the larvae of *Atrina japonica* (Ota, 1961; Ohashi *et al.*, 2008), *A. pectinata* (Ota, 1961), *A. zelandica* (Booth, 1979), *Pinna carnea*, *P. rudis* and *A. seminuda* (Allen, 2011). For all of these, the larvae are of similar distinctive gross morphology and, where reported, share similar hinge structures. However, late-stage larvae are most often the focus of the work, with few mentions of early-stage larvae and no accounts of how hinge structure changes with ontogeny.

The spatial extent of larval transport is governed by the interplay between abiotic factors that affect the horizontal and vertical rate of water movements, and biotic factors such as the vertical movements of larvae and the duration of the pelagic phase (Cowen & Sponaugle, 2009; Selkoe & Toonen, 2011). Determining pelagic larval duration (PLD) requires a means of age estimation. Growth lines, which can be defined as abrupt or repetitive changes in the character of an accreting tissue (Clark, 1974), have been used to estimate the age of adult bivalves (Thompson, Jones & Dreibelbis, 1980; Jones & Quitmyer, 1996; Moltschaniwskyj & Cappo, 2009). Hurley, Tremblay & Couturier (1987) demonstrated that growth lines visible in the larval shells of *Placopecten magellanicus* were deposited on a daily basis and these structures have been used to investigate life-history parameters, such as PLD, in the early stages of other bivalves (Chícharo & Chícharo, 2000, 2001).

Dispersal distances often fall short of those predicted from PLD alone, suggesting that other phenomenon, such as the behaviour of larvae and/or hydrodynamic patterns, can act to limit dispersal (Shanks, Grantham & Carr, 2003; Shanks, 2009). In addition, since oceanic circulation patterns along with the intensity of weather systems vary on a seasonal basis, the timing of spawning can have a direct impact on dispersal potential (Edwards *et al.*, 2007). Although bivalve larvae

have limited swimming ability (a few mm s^{-1}; Chia, Buckland-Nicks & Young, 1984), they can alter their vertical position to varying degrees in response to changes in factors such as light and gravity (Bayne, 1964; Weidberg *et al.*, 2015), hydrostatic pressure (Bayne, 1963), temperature, salinity and food concentration (Raby *et al.*, 1994; Pearce *et al.*, 1996; Dobretsov & Miron, 2001), tidal conditions (Knights, Crowe & Burnell, 2006) and wind-induced turbulence (Weidberg *et al.*, 2015). Such small changes in vertical distribution can affect large scale horizontal transport in a flow field where there are vertical differences in current speed and direction (Edwards *et al.*, 2007; Corell *et al.*, 2012; Weidberg *et al.*, 2015). Obtaining such information for bivalve species of conservation importance is a prerequisite for informing management through spatially explicit tools such as marine protected areas (MPAs).

Here we aim to: (1) identify and describe the early- and late-stage larvae of *A. fragilis*; (2) determine the time of *A. fragilis* spawning; (3) estimate the PLD of the larvae and (4) assess any vertical distribution preference displayed by the larvae in relation to the physical structure of the water column.

MATERIAL AND METHODS

Plankton sampling

Zooplankton samples were collected from the west coast of Scotland (Fig. 1) using a 1-m diameter bongo, paired zooplankton net (both having a mesh of 250 µm in the nets and of 200 µm in the cod-ends), which was towed obliquely through the water column. Sampling was conducted at stations 1 – 7 (haul numbers A14089:102) during the first week of October 2014 and stations 8 – 13 on 10 and 11 February 2015 (haul numbers SJM001:012). One replicate from each paired sample was preserved in 100% ethanol and the other in 80% isopropanol. The replicates preserved in ethanol were retained for molecular analysis, while bivalve larvae were separated and counted from the isopropanol-preserved samples using light microscopy. Samples are stored at Marine Scotland Science's laboratory, Aberdeen.

Molecular analysis

From the ethanol-fixed material for the seven stations in October 2014, three separate 5-mm^3 replicate samples (A – C) were taken from each, resulting in 21 replicates. DNA was extracted independently from the replicates using the DNeasy Blood and Tissue Extraction kit (Qiagen), following the manufacturer's protocol, with overnight digestion (10-16 h) at 56 °C and 500 rpm on a ThermoMixer (Eppendorf). The extracted DNA solutions were quantified by Nanodrop (LabTech) and diluted to 25 ng/µl in order to obtain the desired amount of template DNA (50 ng) for PCR amplification.

Cytochrome *c* oxidase subunit I (COI) primers LCO_{1490} and HC_{02198} (Folmer *et al.*, 1994) were used as a positive control to amplify a 710-bp fragment of the COI

gene across many invertebrate species. *Atrina*-specific primers were designed by aligning all available COI sequences of *Atrina* species in GenBank, using CLC Genomics Workbench (CLC bio). As none of the available sequences were from UK specimens (all came from the Mediterranean Sea), primers were designed in the most conserved regions found across *A. fragilis* and *A. chautardi*, the closest relative to *A. fragilis* (Lemer *et al.*, 2014). These primers (AfrCOI-F01, AfrCOI-R01, AfrCOI-R02 and AchCOI-R03 (Table 1), were designed to amplify a 421-bp fragment of the COI gene, including the primer sequences (Integrated DNA Technologies). The forward primer's location was in a region that is fully conserved between both species. The reverse primer, however, was located in a region that contained two mismatches between the species. Therefore, two species-specific reverse primers, AfrCOI-R02 for *A. fragilis* and AchCOI-R03 for *A. chautardi* were designed. The third reverse primer, AfrCOI-R01, included two degenerate sites, with each site being an equimolar mixture of two different nucleotide bases (Table 1). This design compensated for possible mismatches and facilitated primer annealing to UK *A. fragilis* DNA templates in the event that base pairs were variable at the two heterogeneous sites. As no noticeable differences were observed between the three different combinations of the specific primers tested in an initial PCR amplification, reverse primer AfrCOI-R01 was used in all subsequent PCR amplifications along with forward primer, AfrCOI-F01. The PCR protocol used by Lemer *et al.* (2014) was followed for both the universal invertebrate and newly designed specific primers. A negative control of distilled water was included in all PCR amplifications to ensure the PCR amplification was free from DNA contamination. If the negative control gave a positive signal, the PCR amplification was deemed invalid. Positive controls comprised of DNA extracted from four Greek adult *A. fragilis* specimens were also

included to check that PCR conditions successfully amplified the target template DNA.

PCR products were visualized using E-Gel agarose SYBR Safe gels (2% agarose) (Invitrogen, Life Technologies). PCR products that produced a band of the correct 421-bp size were subsequently purified using Rapid PCR Cleanup Enzyme Set (New England Biolabs), following the manufacturer's protocol. Sequencing reactions were then carried out using AfrCOI-F01 and AfrCOI-R01 as sequencing primers, before running the PCR products on a Capillary Sequencer (ABI 3730) (Life Technologies). The sequence chromatograms were visualized and edited using CodonCode Aligner (CodonCode). Basic local alignment search tool (BLAST) sequence similarity was then searched against the GenBank database to confirm that the generated sequences matched previously submitted *A. fragilis* sequences over the targeted COI region.

Morphological analysis

Larvae whose gross shell morphology matched the general description of pinnid larvae were measured (length L and height H, i.e. the axes parallel and perpendicular to the hinge line, respectively) and their hinge structure examined. Measurements were conducted on light microscopes that were calibrated and tested for measurement error. In order to observe the hinge apparatus, the valves of the larvae were disarticulated and the soft tissues removed, by immersing the larvae in an 8 – 10% sodium hypochlorite solution for a few minutes, before rinsing in filtered seawater. Hinge structure and growth lines were investigated using Nomarski differential interference contrast microscopy (DIC) and scanning electron microscopy (SEM). Shells were then mounted on stubs and sputter coated in gold before

examination in the SEM (Zeiss EVO MA10, operating in backscatter mode). Growth lines were interpreted using the description of Hurley *et al.* (1987). Right and left valves were distinguished by defining the anterior and posterior margins of the larval shell. Pinnid larvae are characteristically elongated anteroventrally, with the anterior shell margin being straighter than the posterior one (e.g. Booth, 1979; Malchus, 2004; Allen, 2011) and the provincular teeth of pinnids are inequilateral with the anterior series consisting of smaller, more numerous and even teeth than those of the posterior series (Booth, 1979; Malchus, 2004; Malchus & Sartori, 2013). Pinnid larvae are heteromyarian in nature, i.e. two adductor muscles are present, with the posterior being rounder, larger and further from the valve margin than the anterior (Allen, 2011).

Time-series study

Additional zooplankton samples were collected between April 2014 and September 2015 on the *MV Lochnevis* Caledonian MacBrayne passenger ferry when operating between the Isles of Rum and Canna (Fig. 1). A total of 83 samples were collected (Table 2) using a custom-built plankton sampler, consisting of a 200-µm mesh cod-end inside a 68-µm cod-end, that was fitted to one of the ship's seawater pumps (capacity 480 l min^{-1}). Samples were preserved in 100% ethanol for later analysis.

Depth-stratified study

Depth-stratified sampling was used to assess the vertical distribution of larvae in the SoC on 27 and 28 July 2014 (Fig. 1). An OCEAN (opening–closing environmental acoustic net) sampler (OS), comprised of seven individual 200-µm mesh nets and cod-ends, collected six samples at seven depth intervals (D1: 180 – 150 m, D2: 150

– 120 m, D3: 120 – 90 m, D4: 90 – 60 m, D5: 60 – 30 m, D6: 30 – 15 m, D7: 15 – 0 m) and at three different times of day (early morning, mid morning and afternoon) and during both ebb and flood tides. Water-column profiles were taken with a Sea-Bird SBE 19plus profiler CTD immediately after each OS tow (apart from a 107-min gap between the OS1 and CTD1 samples due to a technical issue). All OS and CTD samples were collected from within a 4 km x 350 m rectangular area. Tidal data were obtained for the closest tidal gauge site to the SoC (Tobermory) in the UK Tide Gauge Network, through the British Oceanographic Data Centre and adjusted for tides in the SoC.

RESULTS

Molecular analysis

The universal primers consistently amplified a 710-bp fragment (amplicon) from all samples apart from sample 5 (Fig. 2A). The more specific primer pair amplified the expected 421-bp fragment from the positive controls and samples as well as samples 1, 4 and 7. Replicate 7C consistently produced the strongest band, though no band was detected for 7A (Fig. 2B). The band strength on the gels was qualitatively assessed to check whether both sets of primers produced an amplicon for a particular replicate (Table 3). PCR products that showed bands for the more specific primers were analysed to obtain DNA sequences for the COI barcode region. In total, eight replicates and the four positive controls were sequenced with both the forward and reverse specific primers. The sequences were then trimmed and compared with COI sequences in the GenBank database (Table 4). The forward primer sequences of controls 3, 4 and 5 had 100% sequence similarity to *Atrina fragilis* sequences found in GenBank (Table 4). The forward primer sequence for control 1 had one mismatch with the three other controls. Of the remaining replicates, 7C had 100% similarity to *A. fragilis* sequences found in the database for both forward and reverse primers (Table 4), indicating the presence of *A. fragilis* larval DNA. The forward primer sequence was identical to the control 3, 4 and 5 forward primer sequences, with the consensus sequence having no mismatches.

Morphological analysis

No late-stage pinnid like larvae were collected in the October sampling, though molecular analysis confirmed the presence of *A. fragilis* DNA during this sampling

period. There were, however, a range of early-stage larval bivalve types, whose gross morphology broadly corresponded to that of pinnid larvae, i.e. they were distinctly triangular, so these were separated for further investigations. Larvae of different types were subjected to further molecular analysis, but unfortunately DNA extraction of these samples was unsuccessful. Morphological analyses revealed a single larval type that most closely resembled those of pinnids. While the strength of a PCR product band does not necessarily reflect the abundance of larvae in a sample, it is noteworthy that the strongest PCR product band and only 100% match with the GenBank sequences for *A. fragilis* was observed in sample 7 (replicate 7C), where this type was most abundant. These earlier-stage larvae (length < 400 µm) are distinctively triangular in shape (Figs 3B, C, 4A). The distinctive umbos are higher than other larvae of similar length and, while not as pronounced as the later-stage larvae, are of the same character (Fig. 3C, F). Although hinge structure is strictly comparable only between larvae at the same developmental stage (Rees, 1950), the hinge structure of these earlier stage larvae is of the same type as the later-stage ones, with the anterior series consisting of fewer and more robust teeth than the posterior series (Fig. 3D). The series of posterior teeth extends along the posterodorsal axis during development, as shown by the formation of small teeth at the posterior edge of the posterior series (Fig. 3D). The colour of the earlier-stage larvae is comparable to the later-stage ones, with the anterior and posterior abductor muscles clearly visible through the shell (Fig. 3A). No pigment spots were observed in larvae at any development stage.

Late-stage *A. fragilis* larvae (Fig 4A, F, G) were only collected in February in samples 10A, 12A & B and 13A, with nine larvae collected in total. The largest of these had L of 622 µm and H of 558 µm (Fig. 3A). These larvae are comparable with

326 descriptions of other late-stage pinnid larvae in the literature (Ota, 1961; Booth,
327 1979; Ohashi *et al.*, 2008; Allen, 2011). The valves of late-stage (length > 400 µm)
328 *A. fragilis* larvae are distinctive in terms of their size and triangular shape (Fig. 3F,
329 G). The valves are inequilateral, having a straight to slightly concave anterodorsal
330 shell margin and a convex posterodorsal margin. The distance from the midline to
331 the anterodorsal shell margin in the ventral margin area is greater than that to the
332 posterodorsal margin, giving the larvae a somewhat 'pinched' appearance at the
333 distinctively prominent, 'knobby' umbos. The anterior and posterior abductor
334 muscles, which are bean-shaped and circular, respectively, in cross-sectional profile,
335 are clearly visible through the transparent and pale golden shell (Fig. 3A, G). The
336 larval hinge structure is comprised of a thickened provinculum with 4 – 5 simple
337 rectangular taxodont teeth lying posterior to the umbo, and 6 – 7 teeth lying anterior
338 to the umbo, with an undifferentiated central region (Fig. 3E). The anterior teeth are
339 larger and more robust than the posterior series. The ligament lies anterior to the
340 posterior teeth. The first prodissoconch (small circular valves without growth lines) is
341 distinct at the umbo for both the late- (Fig. 3E) and early-stage (Fig. 3C, D) larvae.
342 Growth lines showed a similar hierarchy in prominence to that described by
343 Hurley *et al.* (1987), with major growth lines, previously found to correspond to daily
344 increments, being more prominent. Major growth lines on the disarticulated right
345 valve were counted from 13 *A. fragilis* larvae (representing the observed size range)
346 at a focal plane similar to that described by Hurley *et al.* (1987) (Fig. 4). Major
347 growth-line counts ranged from 27 – 94 over larval L of 217 – 620 µm (Fig. 5). Major
348 lines lay on average 7 µm (range = 4.2 – 9.9 µm) apart, with minor lines lying on
349 average 2.2 µm (range = 1.3 – 3.2 µm) apart. The number of major growth lines

corresponded well to larval L when a simple linear model was fitted to the data (adjusted R^2 = 0.96, y = 6.19, P < 0.001).

Time-series study

A total of 3,086 bivalve larvae were collected from the time-series samples, with 12 early-stage *A. fragilis* larvae identified. The mean density of bivalve larvae per m^3 of seawater was lowest in samples taken from January to March (< 1.75 $n \cdot m^{-3}$), and then increased steadily to a peak in August (215 $n \cdot m^{-3}$), before dropping again over autumn and winter, closely following the annual cycle in water temperature (Table 1, Fig. 6). March sampling collected a total of five bivalve larvae, including one *A. fragilis* larva (L = 360 µm, H = 350 µm), resulting in March having the highest proportion of *A. fragilis* larvae despite it being one of the most poorly sampled months (Table 1). In terms of density per m^3 of seawater, *A. fragilis* larvae were most prevalent in samples taken during the summer months, particularly in August (0.63 $n \cdot m^{-3}$) and September (0.5 $n \cdot m^{-3}$), and were also detected during June (0.25 $n \cdot m^{-3}$) and July (0.1 $n \cdot m^{-3}$) (Fig. 6). There is evidence that spawning occurs over winter with larvae detected in November (0.17 $n \cdot m^{-3}$) and March (0.33 $n \cdot m^{-3}$), as well as in the February samples.

Depth-stratified study

The pycnocline was consistently observed at a depth of between 40 – 75 m during all sampling times, with a tendency to become more pronounced and shallower as the day progressed (Fig. 7A–C). A total of 5,087 bivalves, including eight early-stage *A. fragilis* larvae, were collected in the OS samples. The general trend of increasing density of bivalve larvae from the deepest depth interval 150 – 180 m (D1) to the 30

– 60 m depth interval (D5) was apparent in all sampling groups (Figs 7, 8). When the samples were grouped by time of day (Fig. 7), the afternoon group had the highest densities of bivalve larvae in the 30 – 60 m (D5) depth range, followed by the mid-morning group, with the early-morning samples having the lowest (Fig. 7D). Larval densities from the early-morning samples were more heterogeneously distributed across the range sampled depths than the other two groups. *Atrina fragilis* larvae were observed in the mid-morning and afternoon samples between 30 – 90 m depth (D4 – D6). A similar pattern was observed when the samples were grouped by tidal state (Fig. 8), where OS 2 and 6 and OS 4 and 5 samples were grouped into ebb- and flood-tide groups, respectively. During flood tide, bivalves were collected at higher densities in the deeper samples (60 – 180+ m, D1 – D4) and at lower densities in the shallower samples (< 60 m, D5 – D7) than during ebb tide (Fig. 8C). However, this general pattern when grouped by tidal state does not hold for *A. fragilis* larvae, which were found at higher densities in shallower depths (15 – 30 m, D6) during flood tide.

DISCUSSION

Here we present the first description of larvae belonging to the fan mussel, *Atrina fragilis*, along with details of its early life history. Larvae matching the characteristics of late-stage pinnid larvae were found in samples collected in February 2015. Given that *A. fragilis* is the only member of the Pinnidae found in UK waters, these larvae can be positively identified as belonging to this species. Pinnid larvae, in general, achieve a notably large size when compared with most other extant bivalves (Rees, 1950; Ota, 1961; Booth, 1979; Malchus, 2004; Allen, 2011). The lengths of *A. fragilis* larvae collected in February were characteristically large, but lay towards the upper end of those reported for late-stage pinnid larvae. Ota (1961) reported shell lengths of 400 µm and 600 µm, for *A. japonica* and *A. pectinata*, respectively, while Ohashi *et al*. (2008) reported a length of 514 ± 25.9 µm at settlement for *A. pectinata*. Booth (1979) reported a length of 250–400 µm for *A. zelandica*, with late-stage larvae being > 350 µm, but noted that settling size is apparently highly variable, with larvae up to 800 µm in length encountered in the plankton. Allen (2011) similarly reported a length of about 400 µm for late-stage pinnid larvae.

The literature on pinnids does not describe how morphology or hinge structure change with ontogeny, making the identification of early-stage larvae more difficult. However, concurrent molecular and morphological analyses indicated that early-stage *A. fragilis* larvae were present in the water column during late summer and early autumn. Morphological analysis identified one larval type that most closely matched pinnid characteristics. These larvae possessed a similar hinge morphology and umbo character to the late-stage larvae and were of similar colour. The gross morphology of these early-stage larvae (length < 400 µm), while distinctively

triangular, lacked the characteristic pinched appearance of larvae > 400 µm in length. Hence, it appears that in *A. fragilis* larvae > 400 µm in length, preferential growth occurs towards the anterioventral margin of the larval shell as the papilliform umbo extends dorsally, resulting in the distinctive shape of the late-stage larvae. This asymmetric growth may allow the entirety of the retracted vellum to be accommodated within the larval shell (Allen, 2011).

The ferry-collected time-series data, along with February bongo samples, suggest that *A. fragilis* follows the same pattern of spawning that is found in other pinnids at temperate latitudes, where periods of peak spawning occur over summer and winter while trickle spawning continues throughout the year (Booth, 1979; Qiu *et al.*, 2000; Soria, Pascual & Fernandez Cartes, 2002; Maeno *et al.*, 2009). The length-distribution of the collected larvae help to substantiate this spawning pattern, with larval length increasing from early summer as the year progresses: the largest larvae were collected in February (bongo samples) and March (ferry samples). The ferry sampler was sporadic in capturing bivalve larvae, particularly on a week-to-week basis, where density in consecutive samples often varied considerably. In addition to natural variability, other reasons for this temporal variation are likely due to the sampling being restricted to the uppermost layers of water, combined with possible variation in vertical position of larvae during ontogeny (Cragg, 1980), or in response to water column structure (Raby *et al.*, 1994) or tidal flow (Knights *et al.*, 2006).

No reports on the depth distribution of pinnid larvae are available in the literature, although Allen (2011) collected pinnid larvae from depths of 150 – 200 m. Here, the depth distribution of *A. fragilis* larvae collected by the OS followed the pattern found for bivalves in general in the samples. Bivalve larvae have been

450 observed to aggregate around the pycnocline when the water column is stratified
451 and to be more evenly distributed when the water column is well mixed (Tremblay &
452 Sinclair, 1990; but see Raby *et al.*, 1994). In this study, daily stratification patterns in
453 the SoC, presumably due to seasonal solar heating of surface layers, were
454 coincident with higher aggregations of bivalve larvae above the more defined
455 thermocline later in the day. Such behaviours could help explain the change in
456 relative densities of bivalve larvae in the upper 60 m of the water column throughout
457 the day. When the OS samples were grouped by tidal state, the pattern for *A. fragilis*
458 larvae differed from that of bivalves in general, with higher densities at shallower
459 depths during the flood tide, similar to that observed for mytilid larvae in the southern
460 Irish Sea (Knights *et al.*, 2006). Given the low total numbers and densities of *A.*
461 *fragilis* larvae taken during the depth-stratified sampling, these results suggest that
462 *A. fragilis* larvae may vary in their depth distribution in response to the depth of the
463 pycnocline, time of day and/or tidal flow direction, but do not allow an understanding
464 of the underlying causal mechanism.

465 While no verification of increment periodicity of growth lines was possible,
466 assuming that major growth lines are deposited on a daily basis (as by Hurley *et al.*,
467 1987), the larval shell grew in the region of 6 µm d^{-1}. This is comparable with other
468 bivalve larvae in similar temperature regimes. Hurley *et al.* (1987) reported a growth
469 rate of 3 µm d^{-1} for *Placopecten magellanicus* at 14 °C with linear growth rates, while
470 Sprung (1984) reported a growth rate of 8.1 µm d^{-1} for *Mytilus edulis* at 12 °C, also
471 with linear growth. As *A. fragilis* larvae in Scottish waters experience an average
472 annual temperature range of 7 – 14 °C (Berx & Hughes, 2009), these figures suggest
473 that an estimated growth rate of 6 µm d^{-1} is reasonable.

No estimates for the PLD of *A. fragilis* larvae exist. Using archived images of *A. fragilis* spat taken by Oliver *et al.* (2016) (Fig. 3H), size at settlement can be estimated from larval shell markings, which remain clearly visible through the juvenile shell. This juvenile specimen was collected further south in warmer waters around the Isles of Scilly, UK (50 °N) with measurements of the larval shell suggesting a length of 770 µm at settlement that, based on the growth rates derived for the more northerly captured larvae, translates into a PLD of around 4 months. However, an inverse relationship between shell length at metamorphosis and temperature exists for many bivalve species, where larvae tend to settle at smaller sizes in warmer waters (Lutz & Jablonski, 1978; Cragg & Crisp, 1991). Therefore, any inferences based on length at settlement from the Isles of Scilly larva may underestimate length at settlement for larvae in cooler areas further north. Nevertheless, this estimate of PLD is significantly longer than reports for other pinnid larvae—though not unexpected, as growth rate in bivalve larvae is directly proportional to temperature (Sprung, 1984). At 57 °N, *A. fragilis* in the SoC is towards the most northerly edge of its distribution; the reported PLDs for other pinnids come from latitudes between 40°N and 40°S where average yearly water temperatures range between 15 – 30 °C (Maeno *et al*., 2009). Booth (1979) estimated *A. zelandica* to have a larval duration in the region of 1 month, from the seasonal abundance in plankton samples. More specific accounts of larval duration in pinnids come from aquaculture studies of *A. pectinata,* with a larval duration of 23-47 d (Lin *et al.*, 1987; Ohashi *et al.*, 2008). Such protracted development for *A. fragilis* larvae in the northeast Atlantic may have implications for its population dynamics in this region, where both larval mortality and dispersal are likely to be high due to the extended PLD (Widdows, 1991; Gallego *et al.*, 2016). We speculate that

these life-history features, together with the vulnerability *A. fragilis* to mobile bottom-fishing gears (Solandt, 2003; Fryganiotis *et al.*, 2013) and the industrialization of fishing, may have contributed to the present rarity of this species in the northeastern Atlantic region. Future work on *A. fragilis* should attempt, through treatment with compounds that mark the larval shell, to verify that the periodicity of major growth lines is indeed daily. Such verification would allow for greater certainty when estimating the potential levels of connectivity between suitable habitat areas and areas chosen for conservation of this species.

Currently there are two marine protected areas (MPAs) in the OSPAR network that include *A. fragilis* as a feature identified for protection; the South-West Deeps (England) and the Small Isles (Scotland) MPAs. The description and details of the early life history of *A. fragilis*, when coupled with information on the spatial distribution of suitable habitat (Stirling *et al.*, 2016) and sea circulation models, will help inform population-level connectivity estimates for this rare species of conservation concern, and may highlight new areas suitable for designation as MPAs.

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738 TABLES

739 **Table 1.** Primer sequences (5' end to 3'), direction and length (bp). The reverse
740 AfrCOI-R01 primer sequence has two degenerate sites, with the different nucleotide
741 bases at each site shown in brackets; in each case, the first base is found in *A.*
742 *fragilis* sequences, the second in present in *A. chautardi* sequences.
743

Primer	Direction	Sequence	L
LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	25
HC02198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	26
AfrCOI-F01	Forward	TAGAGTAATTATTCGAACTGAGC	23
AfrCOI-R01	Reverse	T [A/T] CGACGCATATT [C/T] TGAGC	19
AfrCOI-R02	Reverse	TACGACGCATATTCTGAGC	19
AchCOI-R03	Reverse	TTCGACGCATATTTTGAGC	19

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Table 2. Sampling effort (N_s), average number of bivalve ($TNB \cdot m^{-3}$) and early-stage *Atrina fragilis* larvae per m^3 of seawater and observed length ranges of *A. fragilis* larvae in µm (LR) collected each month during time-series sampling.

Month	N_s	$TNB \cdot m^{-3}$	*A. fragilis*$\cdot m^{-3}$	LR
January	4	1.75	0	-
February	6	1.33	0	-
March	3	1.33	0.33	360
April	14	2.64	0	-
May	13	8.92	0	-
June	8	18.75	0.25	130 – 140
July	10	24.3	0.1	140
August	8	215.5	0.63	180 – 280
September	2	40.5	0.5	270
October	4	10.25	0	-
November	6	14.33	0.17	210
December	5	11.8	0	-

Table 3. PCR product band strength approximated for all 28 replicates and both positive and negative controls for the universal primers (UP, LCO1490-HC02198) and the more specific primers (SP, AfrCOI-F01-AfrCOI-R01).

Samples	UP	SP
1A	++	+
1B	+++	+
1C	+++	+
2A	-	-
2B	+	-
2C	+	-
3A	+	-
3B	+	-
3C	-	-
4A	+++	+
4B	+++	+
4C	+++	+
5A	-	-
5B	-	-
5C	-	-
6A	+++	-
6B	++	-
6C	+++	+
7A	+++	+
7B	+++	-
7C	+++	+++
Positive control	+++	+++
Negative control	-	-

+++, strongest band strength; +, faint bands; –, absence of bands.

Table 4. Forward and reverse primer sequence results for eight replicates. Only the forward primer sequence was reliable for the four controls. Where present, the primer regions at the 3' end of the sequence were trimmed. Bases that lack resolution at the 5' end of the sequence immediately after the sequencing primer have been omitted. Trace quality was estimated as high, medium or low based on the quality of the peak and the amount of noise present. Sequences were then searched against the GenBank database using BLAST. The GenBank sequence with the best match was recorded along with its identity score.

Sample	Sequencing primer	Sequence length (bp)	Chromatogram trace quality	GenBank match using BLAST (identity score)
Control 1	Forward	345	High	99% *A. fragilis* (343/344)
Control 3	Forward	345	High	100% *A. fragilis* (344/344)
Control 4	Forward	344	High	100% *A. fragilis* (344/344)
Control 5	Forward	349	High	100% *A. fragilis* (344/344)
1A	Forward	181	Medium	No match
1B	Forward	351	Low	83% *A. fragilis* (286/345)
1C	Forward	352	Low	No match
4A	Forward	354	Low	89% *A. fragilis* (306/344)
4B	Forward	354	Low	92% *A. fragilis* (314/343)
4C	Forward	266	Medium	No match
7C	Forward	345	High	100% *A. fragilis* (344/344)
1A	Reverse	249	High	No match
1B	Reverse	249	High	No match
1C	Reverse	249	High	No match
4A	Reverse	258	High	No match
4B	Reverse	252	High	No match
4C	Reverse	279	High	No match
7C	Reverse	334	High	100% *A. fragilis* (333/333)

807 FIGURE CAPTIONS

808

809 **Figure 1.** Location of plankton samples (1 – 13, bongo nets), ferry route (dotted line),
810 ports, and OCEAN sampler and CTD station locations in the Sound of Canna (inset).

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812 **Figure 2.** Visualization on a 2% agarose gel (21 size-selected replicates, 1A-7C) for the
813 universal invertebrate primers (**A**) and more-specific primers (**B**). The positive and
814 negative controls are located in the lanes to the right of the replicates. The right-hand-
815 most lane contains a 100-bp molecular weight ladder (L). The estimated amplicon size
816 (710 bp or 421 bp) is indicated on the left side of the gel.

817

818 **Figure 3.** Images of *Atrina fragilis* larvae. **A.** Change in shell morphology over ontogeny,
819 external view of LV using LM (from top left to bottom right: shell 1: SJM005; shells 2–5:
820 SJM009; shell 6: SJM011) **B.** External view of LV early-stage larva (A14095) using LM.
821 **C.** SEM of early-stage larva (A14101) viewed from right; prodissoconch I is clearly
822 visible (also in **D** and **E**). **D**. SEM of early-stage larva (A14095), internal view of LV
823 hinge. **E**. SEM of late-stage larval hinge morphology (SJM009), internal view of RV. **F.**
824 Late-stage larva (SJM010), external view of LV using LM. **G.** RV late-stage larva
825 (SJM010) using DIC to accentuate growth lines, external view with LM. **H**. *A. fragilis*
826 spat, external lateral view of left side; larval shell outline shown by dashed line
827 (reproduced with permission from Oliver *et al.*, 2016). Scale bars: **A** = 200 µm; **B, C, F,**
828 **G** = 100 µm; **D, E** = 20 µm; **H** = 0.5 mm. Abbreviations: LM, light microscopy; SEM,
829 scanning electron microscopy; LV, left valve; RV, right valve.

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831 **Figure 4.** Counting of major growth lines on right valve of late-stage *Atrina fragilis* larva
832 870 (SJM010), using DIC to accentuate surface texture. Scale bar = 100 µm.

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834 **Figure 5.** Length of *Atrina fragilis* larvae plotted against number of major growth lines.
835 Linear regression (solid line) and 95% confidence intervals (dashed lines).

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837 **Figure 6**. Time-series zooplankton samples collected by the ferry, showing total number
838 of bivalves (TNB) and the total number of *A. fragilis* larvae observed per m^3 for each
839 month. Temperature is also plotted (Berx & Hughes, 2009).

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841 **Figure 7.** Depth-stratified zooplankton sampling, showing water column structure
842 revealed by CTD (**A–C**) and total number of bivalves (TNB) collected and total number

of *A. fragilis* larvae observed per m^3 using the OCEAN sampler, and grouped by time of day (**D**). **A.** Early morning. **B.** Mid-morning. **C.** Afternoon. OS sample number and time of sampling are provided in the plot legends, with the corresponding depth sampling intervals at which the OS samples were taken overlaid.

Figure 8. Depth-stratified zooplankton sampling collected by OCEAN sampler in relation to tidal state (**A, B**) and the total number of bivalves (TNB) and early-stage *A. fragilis* larvae per m^3 grouped by tidal state. In **C**, 2 and 6 were grouped as Ebb, 4 and 5 grouped as Flood.