

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

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3 **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 $6 \mu\text{m d}^{-1}$ 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 $770 \mu\text{m}$ at settlement. Using presumed daily growth lines, this translates into a pelagic larval duration of around 4 months.

INTRODUCTION

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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

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

78 Bivalve larvae are notoriously difficult to identify using classic morphological
79 methods, particularly during their early stages (e.g. Garland & Zimmer, 2002;
80 Hansen & Larsen, 2005). Nonetheless, various early taxonomists attempted
81 systematic classification of bivalve larvae from their morphology (Bernard, 1895;
82 Borisiak, 1909; Lebour, 1938; Rees, 1950; Yoshida, 1956; Miyazaki, 1962), while
83 subsequent researchers produced keys with shape, dimensions, umbo character,
84 colour, hinge-line length and hinge morphology to distinguish between species
85 (Loosanoff, Davis & Chanley, 1966; Garland & Zimmer, 2002). Morphological
86 identification is greatly facilitated by descriptions of sympatric bivalves that allow the
87 comparison of morphological features and by knowledge of the bivalve fauna of an
88 area (Garland & Zimmer, 2002; Rees, 1950). However, greater certainty in larval
89 identification, particularly for the early stages where morphological identification is
90 most challenging, is now possible through genetic approaches (Garland & Zimmer,
91 2002; Larsen *et al.*, 2005; Fonseca *et al.*, 2010). DNA barcoding, a molecular
92 method that uses short species-specific DNA fragments within a particular gene to
93 identify organisms, is an efficient, robust and standardized tool (Hajibabaei *et al.*,
94 2011). This technique requires an established reference library comprising DNA
95 sequences from the same genomic region, so that the unknown query sequence can
96 be identified and validated when compared with sequences generated from closely
97 related species (Yoccoz, 2012).

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

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

107 The spatial extent of larval transport is governed by the interplay between
108 abiotic factors that affect the horizontal and vertical rate of water movements, and
109 biotic factors such as the vertical movements of larvae and the duration of the
110 pelagic phase (Cowen & Sponaugle, 2009; Selkoe & Toonen, 2011). Determining
111 pelagic larval duration (PLD) requires a means of age estimation. Growth lines,
112 which can be defined as abrupt or repetitive changes in the character of an accreting
113 tissue (Clark, 1974), have been used to estimate the age of adult bivalves
114 (Thompson, Jones & Dreibelbis, 1980; Jones & Quitmyer, 1996; Moltschaniwskyj &
115 Cappel, 2009). Hurley, Tremblay & Couturier (1987) demonstrated that growth lines
116 visible in the larval shells of *Placopecten magellanicus* were deposited on a daily
117 basis and these structures have been used to investigate life-history parameters,
118 such as PLD, in the early stages of other bivalves (Chícharo & Chícharo, 2000,
119 2001).

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

126 have limited swimming ability (a few mm s^{-1} ; Chia, Buckland-Nicks & Young, 1984),
127 they can alter their vertical position to varying degrees in response to changes in
128 factors such as light and gravity (Bayne, 1964; Weidberg *et al.*, 2015), hydrostatic
129 pressure (Bayne, 1963), temperature, salinity and food concentration (Raby *et al.*,
130 1994; Pearce *et al.*, 1996; Dobretsov & Miron, 2001), tidal conditions (Knights,
131 Crowe & Burnell, 2006) and wind-induced turbulence (Weidberg *et al.*, 2015). Such
132 small changes in vertical distribution can affect large scale horizontal transport in a
133 flow field where there are vertical differences in current speed and direction
134 (Edwards *et al.*, 2007; Corell *et al.*, 2012; Weidberg *et al.*, 2015). Obtaining such
135 information for bivalve species of conservation importance is a prerequisite for
136 informing management through spatially explicit tools such as marine protected
137 areas (MPAs).

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

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MATERIAL AND METHODS

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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-mm³ 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 LCO₁₄₉₀ and HC₀₂₁₉₈ (Folmer *et al.*, 1994) were used as a positive control to amplify a 710-bp fragment of the COI

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

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

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

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215 *Morphological analysis*

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

226 examination in the SEM (Zeiss EVO MA10, operating in backscatter mode). Growth
227 lines were interpreted using the description of Hurley *et al.* (1987). Right and left
228 valves were distinguished by defining the anterior and posterior margins of the larval
229 shell. Pinnid larvae are characteristically elongated anteroventrally, with the anterior
230 shell margin being straighter than the posterior one (e.g. Booth, 1979; Malchus,
231 2004; Allen, 2011) and the provincular teeth of pinnids are inequilateral with the
232 anterior series consisting of smaller, more numerous and even teeth than those of
233 the posterior series (Booth, 1979; Malchus, 2004; Malchus & Sartori, 2013). Pinnid
234 larvae are heteromyarian in nature, i.e. two adductor muscles are present, with the
235 posterior being rounder, larger and further from the valve margin than the anterior
236 (Allen, 2011).

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238 *Time-series study*

239 Additional zooplankton samples were collected between April 2014 and September
240 2015 on the *MV Lochnevis* Caledonian MacBrayne passenger ferry when operating
241 between the Isles of Rum and Canna (Fig. 1). A total of 83 samples were collected
242 (Table 2) using a custom-built plankton sampler, consisting of a 200- μm mesh cod-
243 end inside a 68- μm cod-end, that was fitted to one of the ship's seawater pumps
244 (capacity 480 l min⁻¹). Samples were preserved in 100% ethanol for later analysis.

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246 *Depth-stratified study*

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

251 – 120 m, D3: 120 – 90 m, D4: 90 – 60 m, D5: 60 – 30 m, D6: 30 – 15 m, D7: 15 – 0
252 m) and at three different times of day (early morning, mid morning and afternoon)
253 and during both ebb and flood tides. Water-column profiles were taken with a Sea-
254 Bird SBE 19plus profiler CTD immediately after each OS tow (apart from a 107-min
255 gap between the OS1 and CTD1 samples due to a technical issue). All OS and CTD
256 samples were collected from within a 4 km x 350 m rectangular area. Tidal data were
257 obtained for the closest tidal gauge site to the SoC (Tobermory) in the UK Tide
258 Gauge Network, through the British Oceanographic Data Centre and adjusted for
259 tides in the SoC.

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RESULTS

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278 *Molecular analysis*

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

297

298 *Morphological analysis*

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

301 period. There were, however, a range of early-stage larval bivalve types, whose
302 gross morphology broadly corresponded to that of pinnid larvae, i.e. they were
303 distinctly triangular, so these were separated for further investigations. Larvae of
304 different types were subjected to further molecular analysis, but unfortunately DNA
305 extraction of these samples was unsuccessful. Morphological analyses revealed a
306 single larval type that most closely resembled those of pinnids. While the strength of
307 a PCR product band does not necessarily reflect the abundance of larvae in a
308 sample, it is noteworthy that the strongest PCR product band and only 100% match
309 with the GenBank sequences for *A. fragilis* was observed in sample 7 (replicate 7C),
310 where this type was most abundant. These earlier-stage larvae (length < 400 μm)
311 are distinctively triangular in shape (Figs 3B, C, 4A). The distinctive umbos are
312 higher than other larvae of similar length and, while not as pronounced as the later-
313 stage larvae, are of the same character (Fig. 3C, F). Although hinge structure is
314 strictly comparable only between larvae at the same developmental stage (Rees,
315 1950), the hinge structure of these earlier stage larvae is of the same type as the
316 later-stage ones, with the anterior series consisting of fewer and more robust teeth
317 than the posterior series (Fig. 3D). The series of posterior teeth extends along the
318 posterodorsal axis during development, as shown by the formation of small teeth at
319 the posterior edge of the posterior series (Fig. 3D). The colour of the earlier-stage
320 larvae is comparable to the later-stage ones, with the anterior and posterior abductor
321 muscles clearly visible through the shell (Fig. 3A). No pigment spots were observed
322 in larvae at any development stage.

323 Late-stage *A. fragilis* larvae (Fig 4A, F, G) were only collected in February in
324 samples 10A, 12A & B and 13A, with nine larvae collected in total. The largest of
325 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

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

352

353 *Time-series study*

354 A total of 3,086 bivalve larvae were collected from the time-series samples, with 12
355 early-stage *A. fragilis* larvae identified. The mean density of bivalve larvae per m^3 of
356 seawater was lowest in samples taken from January to March ($< 1.75 \text{ n}\cdot\text{m}^{-3}$), and
357 then increased steadily to a peak in August ($215 \text{ n}\cdot\text{m}^{-3}$), before dropping again over
358 autumn and winter, closely following the annual cycle in water temperature (Table 1,
359 Fig. 6). March sampling collected a total of five bivalve larvae, including one *A.*
360 *fragilis* larva ($L = 360 \mu\text{m}$, $H = 350 \mu\text{m}$), resulting in March having the highest
361 proportion of *A. fragilis* larvae despite it being one of the most poorly sampled
362 months (Table 1). In terms of density per m^3 of seawater, *A. fragilis* larvae were most
363 prevalent in samples taken during the summer months, particularly in August (0.63
364 $\text{ n}\cdot\text{m}^{-3}$) and September ($0.5 \text{ n}\cdot\text{m}^{-3}$), and were also detected during June ($0.25 \text{ n}\cdot\text{m}^{-3}$)
365 and July ($0.1 \text{ n}\cdot\text{m}^{-3}$) (Fig. 6). There is evidence that spawning occurs over winter with
366 larvae detected in November ($0.17 \text{ n}\cdot\text{m}^{-3}$) and March ($0.33 \text{ n}\cdot\text{m}^{-3}$), as well as in the
367 February samples.

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369 *Depth-stratified study*

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

375 – 60 m depth interval (D5) was apparent in all sampling groups (Figs 7, 8). When the
376 samples were grouped by time of day (Fig. 7), the afternoon group had the highest
377 densities of bivalve larvae in the 30 – 60 m (D5) depth range, followed by the mid-
378 morning group, with the early-morning samples having the lowest (Fig. 7D). Larval
379 densities from the early-morning samples were more heterogeneously distributed
380 across the range sampled depths than the other two groups. *Atrina fragilis* larvae
381 were observed in the mid-morning and afternoon samples between 30 – 90 m depth
382 (D4 – D6). A similar pattern was observed when the samples were grouped by tidal
383 state (Fig. 8), where OS 2 and 6 and OS 4 and 5 samples were grouped into ebb-
384 and flood-tide groups, respectively. During flood tide, bivalves were collected at
385 higher densities in the deeper samples (60 – 180+ m, D1 – D4) and at lower
386 densities in the shallower samples (< 60 m, D5 – D7) than during ebb tide (Fig. 8C).
387 However, this general pattern when grouped by tidal state does not hold for *A.*
388 *fragilis* larvae, which were found at higher densities in shallower depths (15 – 30 m,
389 D6) during flood tide.

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DISCUSSION

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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 $514 \pm 25.9 \mu\text{m}$ at settlement for *A. pectinata*. Booth (1979) reported a length of 250–400 μm for *A. zelandica*, with late-stage larvae being $> 350 \mu\text{m}$, 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 $< 400 \mu\text{m}$), while distinctively

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

431 The ferry-collected time-series data, along with February bongo samples,
432 suggest that *A. fragilis* follows the same pattern of spawning that is found in other
433 pinnids at temperate latitudes, where periods of peak spawning occur over summer
434 and winter while trickle spawning continues throughout the year (Booth, 1979; Qiu *et al.*,
435 2000; Soria, Pascual & Fernandez Cartes, 2002; Maeno *et al.*, 2009). The
436 length-distribution of the collected larvae help to substantiate this spawning pattern,
437 with larval length increasing from early summer as the year progresses: the largest
438 larvae were collected in February (bongo samples) and March (ferry samples). The
439 ferry sampler was sporadic in capturing bivalve larvae, particularly on a week-to-
440 week basis, where density in consecutive samples often varied considerably. In
441 addition to natural variability, other reasons for this temporal variation are likely due
442 to the sampling being restricted to the uppermost layers of water, combined with
443 possible variation in vertical position of larvae during ontogeny (Cragg, 1980), or in
444 response to water column structure (Raby *et al.*, 1994) or tidal flow (Knights *et al.*,
445 2006).

446 No reports on the depth distribution of pinnid larvae are available in the
447 literature, although Allen (2011) collected pinnid larvae from depths of 150 – 200 m.
448 Here, the depth distribution of *A. fragilis* larvae collected by the OS followed the
449 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 $6 \mu\text{m d}^{-1}$. This is comparable with other
468 bivalve larvae in similar temperature regimes. Hurley *et al.* (1987) reported a growth
469 rate of $3 \mu\text{m d}^{-1}$ for *Placopecten magellanicus* at $14 \text{ }^\circ\text{C}$ with linear growth rates, while
470 Sprung (1984) reported a growth rate of $8.1 \mu\text{m d}^{-1}$ for *Mytilus edulis* at $12 \text{ }^\circ\text{C}$, also
471 with linear growth. As *A. fragilis* larvae in Scottish waters experience an average
472 annual temperature range of $7 - 14 \text{ }^\circ\text{C}$ (Berx & Hughes, 2009), these figures suggest
473 that an estimated growth rate of $6 \mu\text{m d}^{-1}$ is reasonable.

474 No estimates for the PLD of *A. fragilis* larvae exist. Using archived images of
475 *A. fragilis* spat taken by Oliver *et al.* (2016) (Fig. 3H), size at settlement can be
476 estimated from larval shell markings, which remain clearly visible through the
477 juvenile shell. This juvenile specimen was collected further south in warmer waters
478 around the Isles of Scilly, UK (50 °N) with measurements of the larval shell
479 suggesting a length of 770 µm at settlement that, based on the growth rates derived
480 for the more northerly captured larvae, translates into a PLD of around 4 months.
481 However, an inverse relationship between shell length at metamorphosis and
482 temperature exists for many bivalve species, where larvae tend to settle at smaller
483 sizes in warmer waters (Lutz & Jablonski, 1978; Cragg & Crisp, 1991). Therefore,
484 any inferences based on length at settlement from the Isles of Scilly larva may
485 underestimate length at settlement for larvae in cooler areas further north.
486 Nevertheless, this estimate of PLD is significantly longer than reports for other pinnid
487 larvae—though not unexpected, as growth rate in bivalve larvae is directly
488 proportional to temperature (Sprung, 1984). At 57 °N, *A. fragilis* in the SoC is
489 towards the most northerly edge of its distribution; the reported PLDs for other
490 pinnids come from latitudes between 40°N and 40°S where average yearly water
491 temperatures range between 15 – 30 °C (Maeno *et al.*, 2009). Booth (1979)
492 estimated *A. zelandica* to have a larval duration in the region of 1 month, from the
493 seasonal abundance in plankton samples. More specific accounts of larval duration
494 in pinnids come from aquaculture studies of *A. pectinata*, with a larval duration of 23-
495 47 d (Lin *et al.*, 1987; Ohashi *et al.*, 2008). Such protracted development for *A.*
496 *fragilis* larvae in the northeast Atlantic may have implications for its population
497 dynamics in this region, where both larval mortality and dispersal are likely to be high
498 due to the extended PLD (Widdows, 1991; Gallego *et al.*, 2016). We speculate that

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

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

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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.

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Primer	Direction	Sequence	L
LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	25
HC02198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	26
AfrCOI-F01	Forward	TAGAGTAATTATTCTGAACTGAGC	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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761 **Table 2.** Sampling effort (N_s), average number of bivalve ($TNB \cdot m^{-3}$) and early-stage
 762 *Atrina fragilis* larvae per m^3 of seawater and observed length ranges of *A. fragilis*
 763 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	-

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778 **Table 3.** PCR product band strength approximated for all 28 replicates and both
 779 positive and negative controls for the universal primers (UP, LCO1490-HC02198)
 780 and the more specific primers (SP, AfrCOI-F01-AfrCOI-R01).
 781

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	-	-

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

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793 **Table 4.** Forward and reverse primer sequence results for eight replicates. Only the
 794 forward primer sequence was reliable for the four controls. Where present, the
 795 primer regions at the 3' end of the sequence were trimmed. Bases that lack
 796 resolution at the 5' end of the sequence immediately after the sequencing primer
 797 have been omitted. Trace quality was estimated as high, medium or low based on
 798 the quality of the peak and the amount of noise present. Sequences were then
 799 searched against the GenBank database using BLAST. The GenBank sequence
 800 with the best match was recorded along with its identity score.
 801

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

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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).

811

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.

830

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.

833

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).

836

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 m³ for each
839 month. Temperature is also plotted (Berx & Hughes, 2009).

840

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

843 of *A. fragilis* larvae observed per m³ using the OCEAN sampler, and grouped by time of
844 day (**D**). **A**. Early morning. **B**. Mid-morning. **C**. Afternoon. OS sample number and time of
845 sampling are provided in the plot legends, with the corresponding depth sampling
846 intervals at which the OS samples were taken overlaid.

847

848 **Figure 8.** Depth-stratified zooplankton sampling collected by OCEAN sampler in relation
849 to tidal state (**A**, **B**) and the total number of bivalves (TNB) and early-stage *A. fragilis*
850 larvae per m³ grouped by tidal state. In **C**, 2 and 6 were grouped as Ebb, 4 and 5
851 grouped as Flood.

852