Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2019) **85**: 319–326. doi:10.1093/mollus/eyz015
Advance Access Publication Date: 7 August 2019

Extraordinarily long development of the Antarctic gastropod *Antarctodomus thielei* (Neogastropoda: Buccinoidea)

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ABSTRACT

Antarctic animals share many traits that are attributed to evolution in a stable, extremely cold climate. Among invertebrates, development is exceptionally slow, making observational studies of development logistically challenging, particularly when conducted under natural conditions in the field. Using multiple deployments to McMurdo Station, Antarctica, we characterized the development, in the field, of an unidentified buccinoidean gastropod species with encapsulated development. Thirteen egg capsules collected at Granite Harbor, McMurdo Sound, Ross Sea, were attached to natural rock and outplanted at a depth of ∼25 m at the base of the McMurdo Intake Jetty on 2 December 2007, photographed on 5 October 2011 and 6 September 2012 and then returned to the laboratory on 27 November 2015. In 2015, four capsules were open and empty, five were open and contained a single large hatchling and the remaining four capsules were intact but not open, each containing a single large juvenile snail. To identify the developing embryos, we sequenced mitochondrial cytochrome *c* oxidase subunit I (COI) from two hatchlings and compared those sequences with those from adults collected near the egg mass, as well as with sequences of other buccinoideans from GenBank. Based on the close match between hatchling and adult COI sequences (hatchling sequences differed from those of an adult at only 2 of 658 nucleotide positions), we identified the embryos as *Antarctodomus thielei* (Powell, 1958)). The egg mass morphology and development of this species have not been previously described. Our study shows that *A. thielei* has a development time of more than 8 years, which is the longest measured for any gastropod.

INTRODUCTION

The nearshore Antarctic marine benthos is one of the coldest and most thermally stable aquatic environments on Earth (Littlepage, 1965; Clarke, 1998; Cheng & Dietrich, 2007), with conditions that have persisted for more than 4 million years (Pörtner, Peck & Somero, 2007). Animals inhabiting this environment share many unusual traits that are attributed to the constantly cold temperature, including stenothermality, gigantism and slow growth (Clarke, 1983, 1992; Brey *et al*., 1995; Stanwell-Smith & Peck, 1998; Peck & Prothero-Thomas, 2002; Peck, Webb & Bailey, 2004; Peck, Powell & Tyler, 2007; Moran & Woods, 2008; Peck, 2016).

While little is known about the development of most Antarctic invertebrates, those that have been studied develop extraordinarily slowly (reviewed by Pearse, McClintock & Bosch, 1991; Clarke, 1992, 1996; Peck, 2016). Hain & Arnaud (1992) estimated that development of shelled gastropods was up to 30 times slower in the Weddell Sea fauna compared with temperate relatives, with juveniles taking up to 2 years to hatch from egg capsules under laboratory conditions. This represents the longest directly measured development time for any gastropod. There are hints, however, of longer development times in the Antarctic. Moles *et al.* (2017) used the relationships between ovum size, hatching time and environmental temperature for ∼ 70 species of heterobranchs (compiled by Thompson & Jarman, 1986) to estimate that the Antarctic heterobranch *Bathydoris hodgsoni* could spend as long as 9.8 years in the egg capsule. While relationships developed for temperate and tropical heterobranchs must be applied to Antarctic relatives with caution (Moles *et al.*, 2017), if these predictions are accurate, then Antarctic gastropods include some of the longest developmental periods known for any animal.

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Describing the development of Antarctic marine invertebrates is a formidable challenge, particularly for species that are not abundant, reproduce rarely, must be maintained at sub-zero temperatures, and develop in egg capsules for many years. We took advantage of multi-year deployments to McMurdo Station, Antarctica, to follow the development of the egg capsules of an unidentified gastropod species over an 8-year period in the field. Using the mitochondrial gene cytochrome *c* oxidase subunit I (COI), which is widely used for the DNA-based identification of species (Herbert *et al.*, 2003), we identified the juveniles in the egg capsules by generating COI sequence data for two embryos and for adult buccinoideans found at sites close to the egg mass, and comparing these with COI sequence data that are currently available for other Antarctic buccinoideans.

MATERIAL AND METHODS

Collection

Two egg masses were collected by hand while diving using scuba, one at Granite Harbor (77°00.715′S, 162°51.515′E) and the other at Turtle Rock (77°44.639′S, 166°46.175′E) in McMurdo Sound, Ross Sea, Antarctica in November 2007. Masses were found at depths between 20 and 40 m, and were attached to small (~10 cm) loose basaltic cobbles. Rocks with attached capsules were returned to McMurdo Station in a cooler filled with chilled sea water, and capsules were maintained in running seawater at approximately −1.0 °C until opened or outplanted. Based on the size, shape and colour of the egg capsules, it was apparent that the two masses originated from the same species, likely a large gastropod. Therefore, we collected adults from each of two potential parents at the Granite Harbor site. One was a large smooth cream-coloured buccinid that we identified as *Neobuccinum eatoni* (E.A. Smith, 1875). The other was a slightly smaller, finely ribbed snail (also cream-coloured), with a more pronounced siphonal canal and slightly hairy perisotracum, which was subsequently identified as *Antarctodomus thielei* (Powell, 1958). In 2016 we also collected two individuals identified as *Trophonella longstaffi* (E.A. Smith 1907) which, based on photographs, had been considered as a potential parent of the type of egg mass studied by us (Brueggeman, 1998). Aside from *Amauropsis rossiana* (E.A. Smith, 1907), a naticid gastropod that typically has a sand-collar egg mass (personal observation), we did not observe any other large caenogastropod species, or other types of caenogastropod capsules, during casual observations on our approximately 300 scuba dives around McMurdo Sound.

Laboratory measurements

At McMurdo Station, adult snails were immediately preserved in 99% ethanol for later molecular analysis. One capsule from the Granite Harbor mass and two capsules from the Turtle Rock mass were gently scraped off their rock (while leaving the rock underwater) with fine forceps and then opened with fine dissecting scissors. The contents were emptied into dishes of chilled seawater held on ice and examined under a calibrated stereomicroscope for staging and photography. We later used ImageJ v. 1.51h (Rasband, 2016) to measure the maximum diameter of each embryo and ten nondeveloping (nurse) eggs from each capsule.

Outplant

The remaining 13 capsules of the Granite Harbor mass were left intact and attached to their rock, and the rock was outplanted at approximately 25 m depth at the base of the McMurdo Intake Jetty (77°51.069′S, 66°39.855′E) on 2 December 2007. For outplanting, the rock with attached capsules was placed in a 1-quart rectangular plastic freezer box, the tops and sides of which had been cut out and replaced with screening to allow water flow, retain hatchlings and exclude potential large predators such as the sea star *Odontaster validus*. The box was opened and capsules were photographed underwater on 5 October 2011 and 6 September 2012; the rock and capsules were subsequently retrieved and brought into the laboratory on 27 November 2015. The entire mass was photographed in the laboratory and the total height, width, peduncle length and basal diameter of 5 of the 13 capsules were measured using digital callipers. Three capsules were then opened with fine dissecting scissors and the contents were photographed; the contents and remaining capsules were preserved in 95% ethanol and brought to the University of Hawaii for measurement and barcoding.

DNA sequencing

DNA was extracted from an approximately 1 mm^3 piece of the foot of two hatchling snails and each adult snail. Each tissue sample was rinsed in distilled water and incubated overnight at 56 °C in 500 µl 2X CTAB extraction buffer with 5 µl of proteinase K (Qiagen). DNA was then extracted twice with 500 µl of chloroform–isoamyl alcohol (24:1) and then precipitated with 1000 µl of cold ethanol (95%). Samples were placed in a freezer (−20 °C) for 1 h to enhance DNA precipitation and then centrifuged at 13000 g for 10 min. The precipitated pellet was washed twice with cold ethanol (70%), dried, re-suspended in 50 µl of water and stored at −20 °C.

A 710 bp fragment of COI was amplified from the extracted DNA using the jgHCO2198 and jgLCO1490 primers (Geller, Meyer, Parker & Hawk, 2013). Reactions were composed of 9.5 µl dH2O, 12.5 µl Taq 2X Ready Mix (Bioline, Taunton, MA), 1 µl of each 10 µM primer and 1 µl of genomic DNA. The amplification cycle consisted of an initial denaturing step at 94 °C for 90 s, followed by 35 cycles of 94 °C for 30 s, 45 °C for 30 s and 68 °C for 90 s (with a ramp to 68 °C of 1 °C/s). Excess primers and nucleotides were removed by incubating with exonuclease I and shrimp alkaline phosphatase (New England BioLabs, Ipswich, MA) at 37 °C for 30 m. Approximately 600–650 bp were then sequenced from each primer on an automated sequencing system (Applied Biosystems) at the Advanced Studies in Genomics, Proteomics, and Bioinformatics core facility, University of Hawaii at Manoa. Complementary sequences were aligned and merged in Geneious v. 9.1.5 (Biomatters, Auckland). Each consensus sequence was compared with available GenBank sequences using BLAST with default parameters on the NCBI website. All new sequences were submitted to GenBank (Table 1).

Phylogenetic analysis

In addition to the new sequences generated in this study, we used CO1 sequences for 14 buccinoideans and 1 muricoidean (used as the outgroup) from GenBank. Also included were four previously unpublished COI sequences of Antarctic buccinoideans from the National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM) collections; these have been generated using protocols identical to those of Harasewych (2014) (Table 1). Sequences were aligned using the MUSCLE plug-in within Geneious. A maximum likelihood (ML) tree (with bootstrap values) and a Bayesian tree (with posterior probabilities) were generated within Geneious using the RaxML and MrBayes plugins, respectively.

Morphological identification

The adult specimen (Fig. **1**B, C) shown to be conspecific with the hatchlings (Fig. 1A) (based on COI sequences) was confirmed as *A. thielei* by comparison with material in the USNM collections (USNM 887954), as well as with the published figure of the holotype (Powell, 1958: pl. 3, fig. 8). The radula of one of the hatchlings was prepared using the technique of Holznagel (1998). The radula,

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Table 1. Sources for the COI sequence data used to produce the phylogenetic trees in Figure 3.

Taxon	Locality	Voucher	GenBank acc. no.	Reference
Trophonella scotiana (Powell, 1951)	Ross Sea, Antarctica	MNA 4	JX110861	Barco *et al.* (2012)
T. longstaffi (E.A. Smith, 1875)	McMurdo Sound, Antarctica	USNM 1468793	MG738675	This study
Buccinulum linea (Martyn, 1784)†	Waitemata Harbour, NZ	NMNZ M.317711	KP694157	Donald *et al.* (2015)
Belomitra paschalis (Thiele, 1925)	Mozambique Channel	MNHN-IM-2009-18853	JQ950229	Kantor *et al.* (2012)
Buccinum undatum (Linnaeus, 1758)†	UK	BMNH 20080004	FN677402	Barco *et al.* (2010)
Columbella mercatoria (Linnaeus, 1758)†	Guadeloupe	MNHN 184659120	KY464895	Russini *et al.* (2017)
Busycon carica (Gmelin, 1791)‡	Woods Hole, MA, USA	USNM 1021638	AY194560	Wise *et al.* (2004)
Neptunea antiqua (Linnaeus, 1758)†		MT00960	KR084936	Barco *et al.* (2016)
Colubraria muricata (Lightfoot, 1786)†	Panglau, Philippines	BAU00629	FM999177	Oliverio & Modica (2010)
Nassarius arcularia (Linnaeus, 1758)†	Maputo Bay, Mozambique	MNHN-IM-2009-22307	KY451340	Galindo *et al.* (2016)
Melongena (Linnaeus, 1758)†	Celestun, Yucatan, Mexico	USNM 1021639	AY194558	Wise *et al.* (2004)
Fasciolaria tulipa (Linnaeus, 1758)†	Guadeloupe	MNHN-IM-2013-19559	KT753954	Couto *et al.* (2016)
Pareuthria fuscata (Brugière, 1789)	Campbell Is. NZ	NMNZ M.317716	KP694137	Donald *et al.* (2015)‡
P. fuscata (Brugière, 1789)	Ushaia, Argentina	BAU00697	FM999174	Oliverio & Modica (2010)§
Neobuccinum eatoni (E.A. Smith, 1875)	Off Princess Elizabeth Land, Antarctica	AAD-57888	HQ918368	Stark & Johnstone (unpublished data)
N. eatoni (E.A. Smith, 1875)	Ross Sea, Antarctica		GU227108	Heimeier, Lavery & Sewell (2010)
N. eatoni (E.A. Smith, 1875)	McMurdo Sound, Antarctica	USNM 1468794	MG738676	This study
Chlanidota signeyana (Powell, 1951)	W of Brabant Is, Antarctica	USNM 1123547	MG738677	This study
*C. signeyana (*Powell, 1951)	N of S Shetland Islands	USNM 1123552	MG738678	This study
Probuccinum tenerum (E.A. Smith, 1907)	W of Adelaide Is., Antarctica	USNM 1121909	MG738679	This study
P. tenerum (E.A. Smith, 1907)	S of Palmer Peninsula, Antactica	USNM 1121966	MG738680	This study
Antarctodomus thielei (Powell, 1958)	McMurdo Sound, Antarctica	USNM 1461012	MG738681	This study
A. thielei Hatchling 1	McMurdo Sound, Antarctica	USNM 1461013	MG738682	This study
A. thielei Hatchling 2	McMurdo Sound, Antarctica	USNM 1461013	MG738683	This study

†Type species of type genus of family or subfamily.
‡Reported as *Pareuthria campbelli* (Filhol, 1880), a junior synonym of *Pareuthria fuscata* (see MolluscaBase, 2017b).
Reported as *Pareuthria plumbea* (Philippi, 1844), a junior synonym of *Pareuthria fuscata* (see MolluscaBase, 2017b).
Abbreviations: BAU, Department of Animal and Human Biology, Rome; MBM, Marine Biology Museum, Chinese Academy of Sciences, Qingdao; MNHN, Museum national d'Histoire naturelle, Paris; NMNZ, Museum of New Zealand, Te Papa Tongarewa, Wellington; USNM, National Museum of Natural History, Smithsonian Institution, Washington, D.C.

shell and operculum were coated with gold and photographed using a Leica Stereoscan 440 electron microscope. The radula (Fig. 1H) and protoconch (Fig. 1E, G) of one of the hatchlings matched those illustrated for *A. thielei* by Numanami (1996: fig. 96).

RESULTS

Laboratory measurements and observations

Individual capsules were yellow, goblet-shaped and pedunculate (Fig. 2A). Capsules measured 10.8± SD 0.2 ($n = 5$) mm in total height and 10.2 ± 0.2 mm in width across the main capsule body; the peduncle averaged 2.0 ± 0.1 mm in height and the base of the peduncle (where it attached to the rock) averaged 7.5 ± 0.2 in greatest width. Capsules were translucent but not transparent, so that details of the contents were not visible through the capsule wall. The capsule wall was extremely tough and difficult to puncture or cut.

The Granite Harbor capsules had no visible biofouling in 2007, suggesting that they were recently laid. The single capsule we opened from this mass in 2007 contained one embryo and many small, round, apparently nondeveloping eggs, the appearance of which was consistent with nurse or trophic eggs (Fig. 2B). Nurse eggs were variable in size, but the largest among them averaged 152.9 μm ± 12.7 ($n = 10$). The embryo (Fig. 2B) was ciliated, 401.3 μm across its maximum diameter and (at an early, preveliger stage of development) lacked a velum, shell, foot or eyes, and the gut did not contain nurse eggs. Each of the two Turtle Rock capsules also contained one embryo and numerous nurse eggs. These embryos were larger and more developed, with a distinct shell, foot and velum, with ingested nurse eggs being visible through the shell (Fig. 2C). The embryos in these two capsules were 1.27 and 1.16 mm in maximum diameter, and these capsules also contained some uneaten nurse eggs.

The remaining 13 capsules from the Granite Harbor mass were intact when outplanted in 2007 (Fig. 2D). When the egg capsules were photographed 5 years later in 2012, all capsules were still intact with no visible signs of hatching (although capsules were visibly more fouled) (Fig. 2E). In 2015, when the capsules were collected and brought into the laboratory, four of the capsules were open at the top and empty; five were open at the top and contained a single large hatchling that very nearly filled the capsule and had not emerged, and the remaining four were still intact and not open, but each contained a single large juvenile snail (Fig. 2F). Unhatched juveniles and hatchlings had finely ribbed white shells (except for the apex, which was smooth) with a thin,

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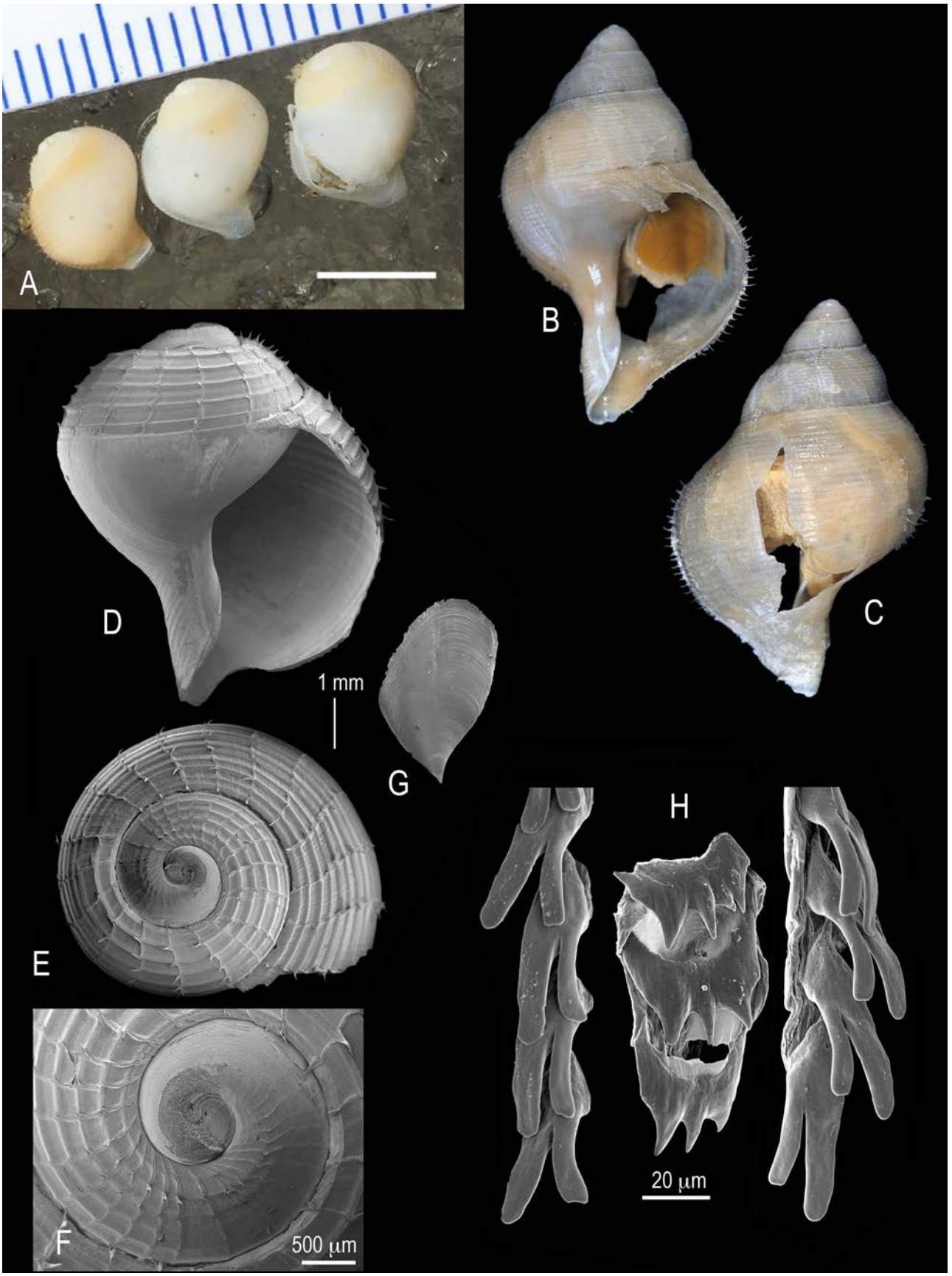

Figure 1. Shell and radular morphology of *Antarctodomus thielei*. **A.** Hatchlings in 2015 immediately after removal from three of the outplanted egg capsules (scale bar = 5 mm). **B**, **C.** Adult specimen, showing apertural (**B**) and dorsal (**C**) views. **D–F.** Shell of hatchling, showing apertural view (**D**), apical view (**E**) and detail of early whorls (**F**). **G.** Outer view of operculum of hatchling. **H.** Radular teeth of hatchling.

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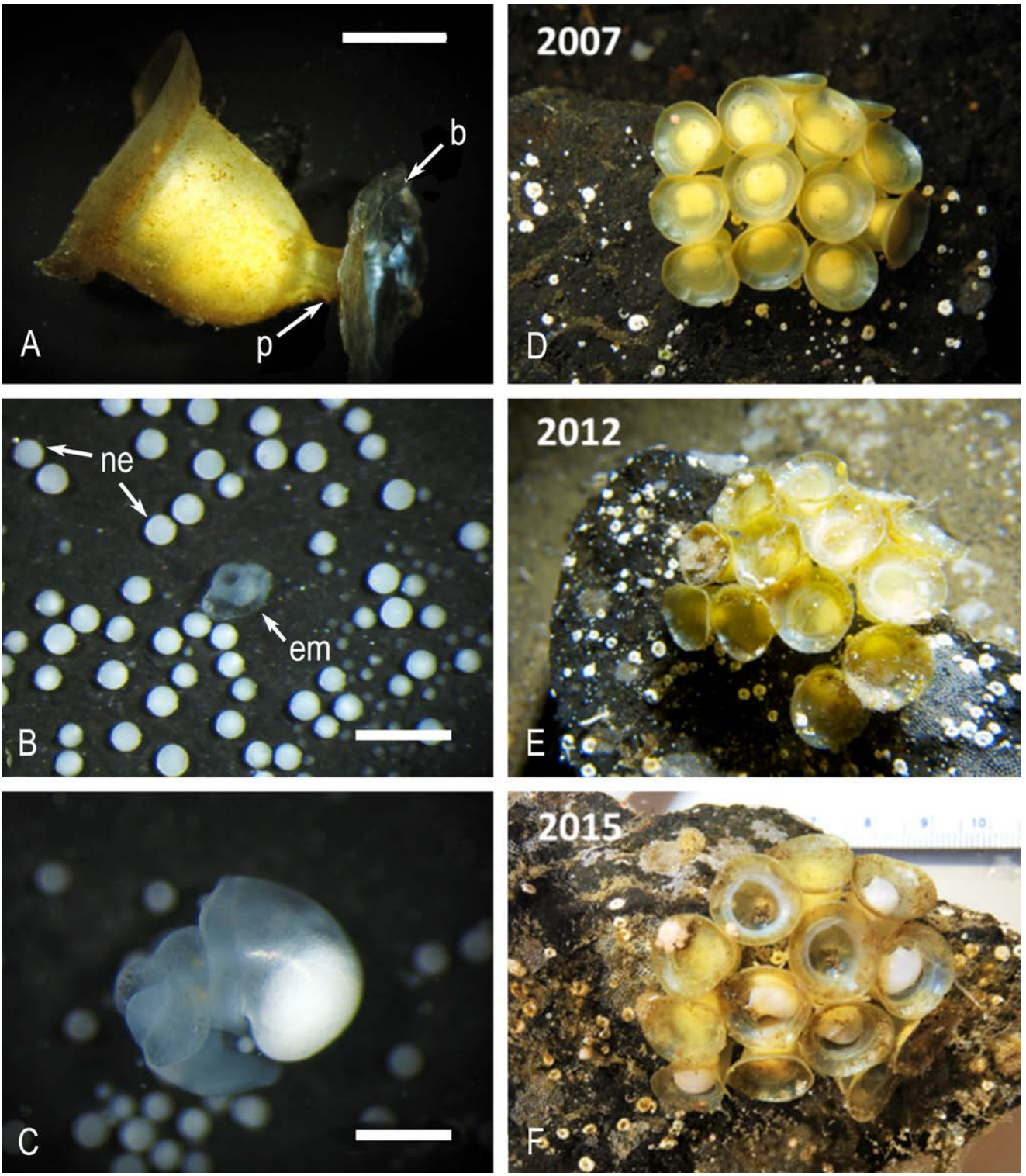

Figure 2. Morphology of the egg mass of *Antarctodomus thielei*. **A.** Individual egg capsule. **B.** Early embryo and nurse eggs from egg mass collected at Granite Harbor (the outplanted mass). **C.** Embryo and nurse eggs from the older Turtle Rock egg mass showing nurse eggs (white mass) inside the larval gut. **D–F.** Egg mass collected and outplanted in 2007. **E.** Egg mass photographed in 2012 in the field. **F.** Egg mass photographed in the laboratory in 2015, immediately after collection. Scale bars: **A** = 1 cm; **C** = 0.5 mm. Abbreviations: b, base; em, embryo; ne, nurse eggs; p, peduncle. Photo credits: **D**, B. Miller; **E**, R. Robbins.

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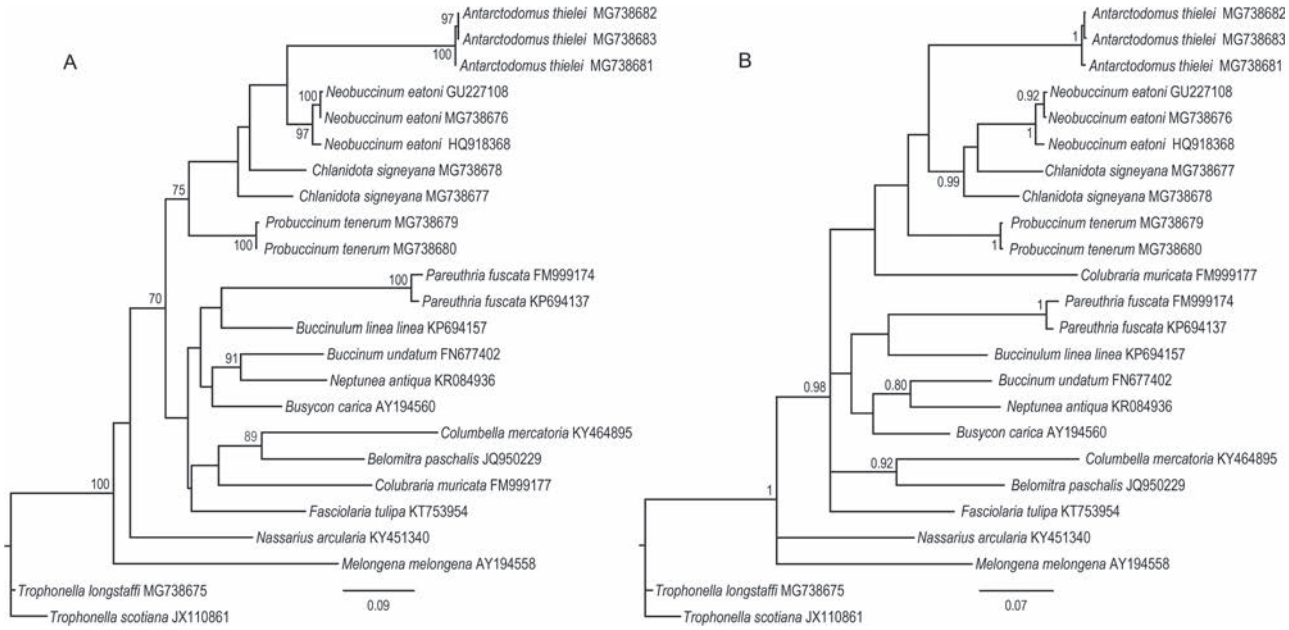

Figure 3. Phylogenetic trees for the species listed in Table 1, as generated using ML (showing bootstrap values ≥70%) (**A**) and Bayesian inference (showing posterior probabilities ≥0.70) (**B**). Scale bars indicate substitutions per site.

hairy, cream-coloured periostracum, white bodies and pigmented eyes that were readily visible through the shell (Fig. 1A). Shell lengths averaged 7.2 mm ± 0.15 ($n = 3$). The hatchlings did not fully emerge from their shells during handling, but hatchlings were clearly alive because they withdrew further into their shells when disturbed.

Molecular phylogenetics

The two hatchlings yielded identical COI sequences that differed only in length of recovered sequence (hatchling 1 = 658 bp, hatchling 2 = 398 bp). Both hatchling sequences differed from that of the adult *Antarctodomus thielei* at only 2 of 658 positions, confirming that they are conspecific. This species, a member of the subfamily Cominellinae on the basis of its distinctive radular morphology (Harasewych & Kantor, 2004), is grouped together with three genera, *Neobuccinum*, *Chlanidota* and *Probuccinum*, of the subfamily Buccinulinae (Harasewych & Kantor, 2004), uniting all buccinoidean samples from within the Antarctic convergence in a clade. This clade is weakly supported (bootstrap value = 75%) in the ML analysis (Fig. 3A), but lacks significant support in the Bayesian analysis (Fig. 3B).

These Antarctic species are included in a clade that contains the type species of six of the eight recognized living families of Buccinoidea (MolluscaBase, 2017a), as well as *Buccinulum linea* (type species of Buccinulinae) and *Pareuthria fuscata* (Columellinae) from the subAntarctic waters off New Zealand and Argentina. This clade has strong (Bayesian analysis, posterior probability = 0.98) to moderate (ML, bootstrap value = 70%) support. The type species of Nassariidae and Melongenidae, the remaining two living buccinoidean families, emerged as sister taxa to this clade (Fig. 3).

DISCUSSION

Compared with the temperate and tropical fauna, little is known about the life histories of nearshore Antarctic molluscs (Arntz *et al.*, 1992). To our knowledge, this is the first study to measure the development time of an Antarctic gastropod in the field, and our record of *c.* 8 years for *Antarctodomus thielei* is the longest documented developmental duration for any non diapausing invertebrate. Both ML and Bayesian trees confirmed that the hatchlings extracted from the egg masses were *A. thielei*, and this allowed us to provide the first description of the egg masses and developmental mode of this species. It is also interesting to note that our ML phylogeny of Buccinoidea (based solely on a portion of the COI gene) recovered clades that reflected geographical distribution, uniting Antarctic samples assigned to the subfamilies Cominellinae and Buccinulinae in a clade separate from Subantarctic Cominellinae and Buccinulinae. However, Cunha, Grande & Zardoya (2009: 11) noted that COI "showed a very low performance in recovering caenogastropod phylogeny" when assessed against phylogenies derived from complete mitochondrial genomes as well as from other individual mitochondrial genes (Cunha *et al.* 2009: table 3).

In general, slow and extended development is a hallmark of the Antarctic marine fauna (Clarke, 1992, 1996; Peck *et al.*, 2006; Peck, 2016), and the gastropods are no exception. Laboratory observations of the nudibranch *Doris kerguelenensis*, and three shelled gastropods, *Neobuccinum eatoni*, *Torellia mirabilis* and *Trophon* cf. *scotianus*, indicated that these species emerge from the egg capsule as juveniles after 21, 15, 24 and 25 months, respectively (Hain, 1992). However, at more than 8 years, the encapsulated development of *A. thielei* is considerably longer than any of these and is more than six times longer than that of *N. eatoni*, which our ML trees showed to belong to a clade of four Antarctic species (i.e. with *A. thielei*, *Chlanidota signeyana* and *Probuccinum tenerum*). Other than the comparatively extended development of *A. thielei*, the two species share a number of striking similarities. Both species undergo direct development, have embryos that feed on nurse eggs in the capsule, are characterized by hatchlings that are similar in size when they emerge from the egg capsule (*A. thielei*, 7.2 mm; *N. eatoni*, 7.5 mm; Hain & Arnaud, 1992) and attain similar adult sizes. In addition, both species occur sympatrically and, at least in McMurdo Sound, share a common thermal regime. Why, then, does *A. thielei* take over 6 years longer to hatch than *N. eatoni*?

Differences in laboratory *vs* field temperatures might, in part, account for this substantial difference. Hain (1992) maintained

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egg capsules in the laboratory at 0 ± 0.5 °C, which is >1 °C warmer than typical water temperatures in McMurdo Sound (Jacobs, Gordon & Ardai, 1979). Antarctic ectotherms are generally highly sensitive to even small changes in temperature (Stanwell-Smith & Peck, 1998), so it is possible that Hain's (1992) comparatively warm laboratory incubations resulted in shorter developmental times than the same species would have had in McMurdo Sound. However, Hain's (1992) data are broadly consistent with our own field outplants of *N. eatoni* at McMurdo (annual temperatures −1.8 °C to −0.5 °C), which showed a similar, and slightly shorter, encapsulated period of approximately 1 year (Moran, unpublished data), so it seems unlikely that Hain's estimates of developmental length for *N. eatoni* are unrealistically short.

The long development of *A. thielei* suggests that its egg capsules are heavily defended, either physically or chemically (Perron, 1981; Strathmann, Staver & Hoffmann, 2002); gastropod egg masses represent high-quality, stationary food sources, and predation on capsules can be very high in some environments (Martel, Larrivee & Himmelman, 1986; Rawlings, 1994). Antipredatory defences can be chemical (McClintock & Baker, 1997) or physical (e.g. thick capsule walls) (Pechenik, 1986; Rawlings, 1994; Turner, Turner & Ray, 2007; Dumont, Roy & Himmelman, 2008; Fukumori *et al.*, 2013). Although we did not test the capsules of *A. thielei* for chemical deterrents or for physical resistance against predators, we found that the capsules were difficult to open in the laboratory, considerably more so than the capsules of *N. eatoni*. Predation rates can be high in the Antarctic benthos (Dayton *et al.*, 1974), but durophagous (crushing) predators have been absent from these communities for millions of years; the primary predators, such as asteroids and nemerteans, generally lack the ability to break through strong physical defences (Aronson *et al.*, 2009). Thus, reinforcement of egg-capsule walls may provide a particularly strong protective benefit in the Antarctic. Reinforced capsule walls, however, may also reduce the permeability of capsule walls to oxygen, which could directly slow embryonic development as a result of hypoxia (Moran & Woods, 2008). Reinforcement could also affect the length of development indirectly, by increasing the safety of embryos and reducing the selective advantages of hatching quickly from the capsule (Strathmann *et al.*, 2002).

There are currently too few data on development and egg capsule morphology of Antarctic gastropods to test these hypotheses, but it is clear that development is slow in general, and is extraordinarily slow for some species like *A. thielei* and the nudibranch *Bathydoris hodgsoni* (Moles *et al.*, 2017). Slow-developing Antarctic species share life-history traits linked to prolonged development, such as long generation time and slow population growth, and these traits confer a high degree of vulnerability to natural and anthropogenic disturbance. As the warming of the Southern Ocean progresses, understanding the factors that have led to long development will shed light on how these taxa are likely to respond to future changes in the physical and biological environment.

ACKNOWLEDGEMENTS

This research was supported by the National Science Foundation (PLR-1341476 to A.L.M. and PLR-1341485 to H.A.W. and B.W.T.). We thank the support staff at McMurdo Station, Antarctica. We thank Yolanda Villacampa (USNM), for assistance with scanning electron microscopy, and we thank Yuri Kantor and Jim McClintock for helpful comments on the manuscript.

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