Vol. 597: 99–113, 2018
https://doi.org/10.3354/meps12569

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published June 11

Reproductive isolation and morphological divergence between cryptic lineages of the copepod *Acartia tonsa* in Chesapeake Bay

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ABSTRACT: Recent advances in molecular technologies have revealed cryptic species across many marine zooplankton taxa. However, the patterns and drivers of cryptic divergence are complex, and few studies have examined reproductive status among lineages through crosses. In this study, we performed pair crosses within and between 2 deeply divergent (cryptic) lineages (named 'fresh' [F] and 'salt' [S]) of the estuarine copepod *Acartia tonsa* from upper Chesapeake Bay, USA, to examine egg production and hatching rate. We also examined differences in morphology (prosome length) and chemical composition of the 2 lineages. Crossing experiments revealed that egg production did not differ among cross types but hatching rate was significantly lower for the between-lineage crosses (mean hatching rate of 0.02 for F×S vs. 0.46 and 0.52 for F×F and S×S, respectively). The nearly complete lack of nauplii production for between-lineage crosses suggests strong reproductive isolation, which supports previous molecular data. Significant differences between the lineages in size (F lineage is 13–14% shorter) and chemical composition (F lineages have 70% less carbon per copepod) may indicate pre-zygotic barriers to reproduction (e.g. morphological or gametic incompatibility). Overall, based on the crossing, morphological, and chemical data reported here, and synthesizing previous biological data on the F and S lineages, we suggest that these cryptic lineages are likely to be separate, reproductively isolated species. Further work examining how divergent lineages of *A. tonsa* respond to environmental change and how they differ in their quality as prey items will be important for understanding trophic dynamics in estuarine environments like Chesapeake Bay.

KEY WORDS: Cryptic species · Zooplankton · Reproduction · Copepod · Food web

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INTRODUCTION

Understanding the patterns and drivers of biodiversity in the world's oceans is a fundamental area of research in marine ecology and biological oceanography (Hutchinson 1961), with implications for geochemical cycling (Lotze et al. 2006, Lomas et al. 2014), food-web dynamics (Turner 2004, Duffy & Stachowicz 2006, Hughes et al. 2008), and the productivity of fisheries (e.g. Beaugrand et al. 2002, 2003). Central to this work is the accurate quantification or delineation of species and species diversity, a process that has historically relied on morphology but can be challenging in species-rich groups or for taxa that lack obvious or reliable morphological characters (e.g. Hillis 1987, Knowlton 1993, Norris 2000, Bickford et al. 2007). Species diversity has been shown to vary substantially among marine fauna (e.g. Tittensor et al. 2010, but see Peijnenburg & Goetze 2013), but whether or not this is due to idiosyncratic features of the biology and ecology of particular taxonomic groups, a lack of data, or bias in the types of taxa examined is not always clear, and species diversity is likely to be substantially underestimated across less-studied marine fauna (Knowlton 1993, Bickford et al. 2007, McManus & Katz 2009).

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For holoplanktonic marine species residing in open and coastal oceans, the paradigm has long been that the relative lack of observable species diversity (morphological conservatism) was due to a combination of factors, including the general lack of geographic barriers, strong ocean currents, and poor swimming ability of plankton that results in high dispersal and high gene flow over large ocean distances (e.g. Norris 2000, Goetze & Ohman 2010, Peijnenburg & Goetze 2013). Application of novel molecular genetic technologies over the last 2 decades has challenged this view of the plankton, with studies repeatedly demonstrating substantial 'unseen' diversity and deep intraspecific genetic divergences among 'populations' of morphologically static groups over ocean-basin scales or even smaller distances (100s to 1000s of kilometers; e.g. Bucklin et al. 1996, Lee 2000, Sáez et al. 2003, Halbert et al. 2013). It is now clear that sibling or cryptic species (genetically divergent clades with similar or indistinguishable morphology) are common among the plankton and zooplankton, having been identified in many pelagic phyla including cnidaria (Schroth et al. 2002, Holland et al. 2004, Warner et al. 2015), foraminifera (Darling & Wade 2008, Aurahs et al. 2009) coccolithophores (Sáez et al. 2003), picoeukaryotes (Slapeta et al. 2006), chaetognaths (Peijnenburg et al. 2006), and crustaceans (e.g. copepods; Bucklin et al. 1996, Lee 2000, Chen & Hare 2008, Goetze & Ohman 2010, Halbert et al. 2013). Varying degrees, rates, and geographic scales of divergence and diversity have been found among and within zooplankton groups (Bickford et al. 2007, Halbert et al. 2013, Peijnenburg & Goetze 2013), suggesting that the underlying drivers of species diversity are complex and that general patterns may be hard to predict. Nevertheless, the existence of cryptic species within such broad taxonomic groups within the plankton suggests even more species diversity than previously thought, and zooplankters have a great propensity to adapt and evolve relatively quickly in response to ecological and anthropogenic change (Peijnenburg & Goetze 2013).

Species diversity and morphological crypsis in copepods

Much research effort has recently been focused on the characterization of species diversity in marine copepods, an ecologically significant group of small crustaceans with high abundances and wide distribution among the world's oceans and coastal seas (e.g. Beaugrand et al. 2003, Turner 2004, Calbet 2008). Previous genetic studies of open-ocean copepod species have revealed striking patterns of restricted gene flow on ocean-basin scales and that many putative 'cosmopolitan' species are actually comprised of multiple, genetically distinct lineages (e.g. Bucklin et al. 1996, Goetze & Ohman 2010, Halbert et al. 2013, Viñas et al. 2015). Consistent with phylogeographic studies of other zooplankton, patterns and scales of divergence appear to vary substantially among cryptic species groups, and sometimes in unpredictable ways. For example, a circumglobal phylogeographic survey of *Eucalanoidea* revealed that while ocean basins often served as boundaries among cryptic lineages, the deeper relationships of sister taxa did not correspond to geographic expectations at the hemispheric level or expectations based on current patterns of ocean circulation (e.g. *Rhinocalanus nasutus*; Goetze & Ohman 2010).

Coastal and freshwater copepod species also show high levels of endemism and crypsis, though sometimes at smaller geographic scales (e.g. Lee 2000, Lee & Frost 2002, Caudill & Bucklin 2004, Marrone et al. 2013). For example, genetic analysis of *Eurytemora affinis* populations across the United States, Europe, and Asia have revealed 8 sibling species with conserved morphology, and crosses have further shown wide variation in the level of reproductive isolation among 'populations' (Lee 2000). Interestingly, reproductive success was greatest among 2 of the more genetically distant clades, suggesting weak correlation between reproductive isolation and genetic distance. These phylogeographic studies highlight the high and likely undersampled diversity of copepods, as well as the complex histories of divergence and biogeography that shape their current genetic (reproductive) interactions. Despite greater appreciation of the extent of species diversity among copepods, the ecological and evolutionary drivers of this diversity and how it varies geographically and across taxonomic groups remains poorly understood.

Cryptic diversity in *Acartia tonsa*

A particularly interesting example of cryptic speciation exists for the calanoid copepod *A. tonsa*, a numerically dominant species that is found in many of the world coastal oceans and estuaries (Razouls 1965) and is tolerant of a wide range of environments and stressors (e.g. González 1974, Brylinski 1981, Cervetto et al. 1999). Initial molecular work using the mitochondrial 16S marker revealed 4 distinct genetic clades with ≥10% divergence across the Northwest

Atlantic, which led authors to conclude that little genetic exchange likely occurred among these lineages (Caudill & Bucklin 2004). Subsequent sequencing of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene, nuclear internal transcribed spacer (ITS) region, and 16S in other studies showed that 3 or more lineages occurred throughout the range of the species in the Western Atlantic and Europe, with phylogenetic structure on the scale of ~1000–2000 km (Chen & Hare 2008, 2011, Drillet et al. 2008a, Chen 2009). The 3 lineages in the NW Atlantic sort by salinity, with a 'fresh' (F) lineage found at salinities below ~10, a 'saline' (S) lineage found primarily from 15 to 25 salinity and an 'intermediate' (X) lineage residing at salinities of 10–22 (Chen & Hare 2008 [their Fig. 1], 2011, Chen 2009). Recent phylogeographic work along the Atlantic coast of South America has also recovered the F and S lineages as well as novel clades (haplotypes) that may represent additional cryptic lineages (da Costa 2011). In Chesapeake Bay, only the F and S lineages have been observed, and these co-occur in the mesohaline and oligohaline salinity zones (Kemp et al. 2005). Concordant divergence data from the mitochondrial *COI* and nuclear ITS regions indicate possible reproductive isolation between these lineages (Chen & Hare 2008).

Phenotypically, there appear to be some differences in morphology, biology, and physiology that likely correspond with the genetically divergent lineages. For example, while adult sizes vary seasonally, S lineage adults of both sexes are always larger than F lineage adults (females are consistently larger than males for both lineages), and less variability in size exists for the X lineage adults (Chen 2009). The presence of deeply divergent lineages may also explain previous observations of substantial phenotypic (biological) differences among samples and experiments. Drillet et al. (2008b) found statistically different rates of development, egg production, and free amino acid pools between 2 mitochondrial lineages with 10–17% divergence (which was not correlated to distance or proximity). Development rates also varied among cultures of *A. tonsa* from Chesapeake Bay (Heinle 1966, Zillioux & Wilson 1966), the Baltic Sea (Berggreen et al. 1988), and southern Europe (Leandro et al. 2006), which may also be linked to divergent lineages found in these systems.

Despite the preponderance of genetic data on divergence and the preliminary observations of phenotypic differences among lineages, experimental work on the status of lineage boundaries and the nature of reproductive isolation among them is lacking for *A. tonsa*. This is not an uncommon situation in the analyses of cryptic species in the marine environment. Broad phylogeographic surveys across ocean basins or coastal populations may not permit further experimental work, and crossing experiments can be time consuming and logistically challenging (but see Lee 2000). However, because genetic divergence is not a reliable predictor of reproductive isolation (e.g. Lee 2000, Edmands 2002), crossing experiments must be performed to directly investigate reproductive status and whether or not viable offspring are produced in crosses between lineages (e.g. the biological species concept; Edmands 2002, Coyne & Orr 2004). Crosses can also provide inference on the nature and possible timing of divergence. For example, if crosses among divergent lineages show hybrid breakdown in the second filial generation (F_2), or complete sterility of the first filial generation (F_1), one can infer something about the extent and nature of post-zygotic isolation (Kozak et al. 2012). Further, if observations of mating behavior can be made (e.g. Goetze & Kiørboe 2008), inferences about pre-mating isolation or mate choice may also be made.

In this study, we examined the reproductive status of the F and S lineages of *A. tonsa* in Chesapeake Bay through a series of crossing experiments, quantifying egg production and nauplii hatching success from within and between lineage crosses. In the process of doing this work, methods were developed to ensure the virgin status of females before crosses and to follow single pair matings over time. In addition, detailed measurements of morphology (e.g. prosome length, mass) and chemical composition (CHN analysis) were made on adults of the 2 lineages to examine potential phenotypic differences and provide additional information to support molecular divergence and reproductive isolation data.

MATERIALS AND METHODS

Collection and culture

Acartia tonsa were collected by plankton tows with a 200 µm mesh, 50 cm diameter ring net at multiple shallow water sites in Chesapeake Bay in summer 2014 and throughout 2015 and 2016 (Fig. 1). To sample for the 'saline' (S) lineage, tows were typically conducted at higher-salinity sites: Muddy Hook Cove (Honga River) on Hooper's Island, MD (38.259756° N, 76.179033° W; 12–15 salinity) and Tyler's Cove (Fishing creek), in Dorchester County, MD (38.351239° N, 76.229659° W; 10–15 salinity). Tows for the 'fresh' (F) lineage were made on the Choptank River at the Horn Point Lab dock, Cambridge, MD (38.593436° N,

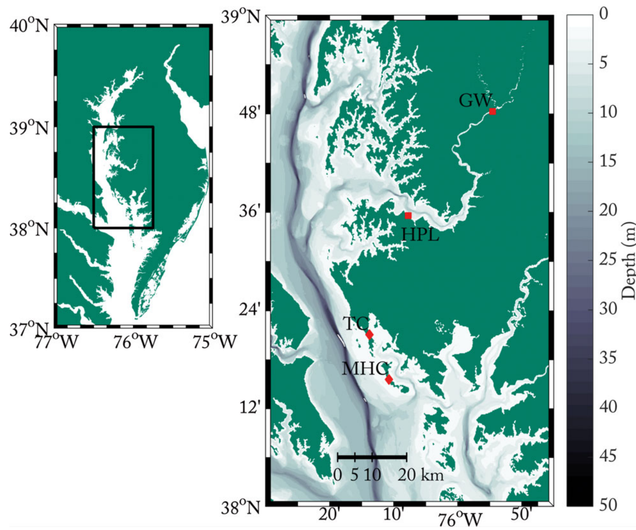

Fig. 1. Map of *Acartia tonsa* sampling sites in Maryland, mid-Chesapeake Bay (enlarged), with inset of the mid-Atlantic region. GW: Ganey's Wharf; HPL: Horn Point Lab; TC: Tyler's Cove; MHC: Muddy Hook Cove

76.128732° W; 7–12 salinity), or (for 'Set 2' experiments) at Ganey's Wharf, Preston, MD (38.804989° N, 75.909184° W; 0.2–3 ppt). Sampling at lower and higher salinities increased the likelihood of getting 'pure' tows of F or S lineages, respectively, facilitating the subsequent creation of lineage-specific cultures used in Set 2 experiments (see below). However, these initial tows typically had a mix of each lineage; thus, the first set of crosses made from these cultures required lineage typing of all adults after crossing experiments (see below). Tows were sorted to remove other plankton and debris, and *A. tonsa* adults were identified under a stereo dissecting microscope and then placed in aquaria vessels (3 l glass pickling jars) at their ambient salinities (8–15) or at intermediate salinities when combining tows (~10–12). Aquaria were maintained on a 12 h light–dark cycle, with feeding of *Rhodomonas salinas* at least every other day and water changes as needed to remove debris, fecal pellets, and excess algae (approximately weekly). Copepods used to determine the lineage composition of initial tows, and adult copepods from Set 1 and 2 crossing experiments were stored in 95% ethanol prior to DNA extraction.

Lineage typing

The genetic lineage of individual copepods (either F or S) was determined using a restriction fragment length polymorphism (RFLP) assay of the mitochondrial *COI* locus (Chen & Hare 2008, Chen 2009). DNA was extracted from individual copepods in 100 µl volumes using either a 5% Chelex solution (e.g. Chen & Hare 2008) or a custom extraction buffer with 0.5% tween, 1× PCR buffer (Promega GoTaq), and 10 mg proteinase K (Plough & Hedgecock 2011, Plough et al. 2014). Chelex extractions were boiled at 98°C for 8 min while proteinase K extractions were incubated at 56°C for 3 h followed by a 15 min boil (95°C); extractions were stored at −20°C until PCR reactions were performed. A 710 bp portion of the *COI* locus was PCR amplified with universal invertebrate primers (Folmer et al. 1994) following the cycle conditions of Chen & Hare (2008) in 25 µl volumes with 4 µl of DNA, 1 unit GoTaq (Promega), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.08 µM of each primer. For the RFLP assay, PCR amplicons from individual copepods were digested with 1–5 units of *HaeIII* enzyme (New England Biolabs), which cleaves (cuts) DNA at 2 positions in the F haplotype that have the *HaeIII* recognition site ('GGCC'), yielding a large fragment of 466 bp and 2 smaller fragments of 140 bp and 104 bp—the S lineage haplotype has no *HaeIII* recognition sites and thus is not digested by the enzyme (Chen & Hare 2008). PCR amplicons of unknown-lineage copepods were digested alongside negative controls (PCR amplifications of water blanks) and positive control amplifications of sequence-verified, pooled F and S lineage DNA, to ensure that the enzyme was active and cut when expected (see Figs. S1–S4 in the Supplement at www.int-res.com/articles/suppl/m597p099_supp.pdf). Digested PCR products were run on 1.5% agarose gels along with 100 bp ladder (and controls), stained with ethidium bromide, and visualized on the UVP Gel Doc-it 2.0 system. A novel, *A. tonsa*-specific *COI* primer set was also developed for lineage-typing copepods in Set 2 experiments and for morphological and elemental comparisons (see below). The amplicon generated is slightly shorter (477 bp), but still retains a *HaeIII* recognition site that is diagnostic for F versus S lineages (355 and 122 bp fragments generated when digesting F haplotypes). PCR with this primer set used the same conditions as the Folmer set described in Chen & Hare (2008) and the sequences are: AtonsaCo1_F (5'-TTG GAG ATG AYC AAA TTT AYA ACG-3') and AtonsaCo1_R (5'-AAA TTT CGG TCK GTT AAY AAY A-3').

Crosses and establishment of pure cultures

Crosses within and between the 2 lineages were performed in 2 sets of experiments that were carried

out in the summer–fall of 2014 (Set 1) and the spring–summer of 2015 and 2016 (Set 2). In the first set of experiments (Set 1), individual copepedites or nauplii were isolated from mixed-lineage cultures and reared in groups (5–10 nauplii) or in isolation until maturity at intermediate salinities (10–12). Once mature, copepods were sexed and a single male and female were placed in 35 mm diameter well plates (Corning Costar 6 well plates) that were fitted with 7.5-cm-tall polycarbonate towers in each well for a total volume of approximately 30 ml of fresh seawater. Crossing experiments were conducted at ~10 ppt. The towers were originally developed for egg production experiments and had 200 µm mesh that was suspended 5 mm from the bottom of the well plate, which allowed eggs to fall through to the bottom of the well but kept adult copepods above, reducing the potential cannibalization of eggs and nauplii (Hopcroft et al. 2005; see Fig. S5 in the Supplement). Tens of pairs were created in this way without prior knowledge of lineage; however, only matings with viable parents (both alive after 24 h) were included in the data analysis. In these 'successful' matings, wells were monitored for the first appearance of eggs, and after 24 h, adults were removed and fixed in ethanol for genotyping to lineage. After adult removal, eggs were counted and returned to the well plate to follow hatching success over the next 48 h. After genotyping adults from each paired cross in Set 1, egg production and hatching rate data could be designated to either the within-lineage cross type, F×F (F lineage male × F lineage female) and S×S (S lineage male × S lineage female) or the between-lineage (hybrid) cross type, F×S (F lineage male × S lineage female) and S×F (S lineage male × F lineage female)

The second set of crossing experiments (Set 2, run in spring–summer 2015 and 2016) used individuals from PCR-validated, 'pure' F and S cultures, which were created from surviving, known-lineage nauplii from within-lineage crosses (from Set 1 experiments) or subsequent tows in low- or high-salinity locations (Fig. 1), from which a portion of the population was verified for lineage via the *HaeIII* RFLP test before being introduced to a given pure lineage culture. Once established, individuals from the F and S cultures were also tested periodically with the PCR–RFLP assay to ensure that only the expected lineage was present (n = 20). The pure lineage cultures were maintained at a common, intermediate salinity (7 ppt) throughout 2015 and 2016 when these experiments were being performed. Periodically, collections of *A. tonsa* from the Choptank River were made to supplement the pure cultures, and in these cases, copepods were slowly stepped up or down in salinity to 7 ppt over a period of days to allow acclimation from their ambient salinities, if necessary.

With pure lineage cultures available, copepodites were collected from each culture and reared in isolation until maturity so that sex could be determined before making pairings. Individuals that were observed to be females were monitored to ensure that they did not produce fertilized eggs and were truly 'virgin' before pairing with males. Because males may attempt mating with late-stage, immature copepodite females (Lonsdale et al. 1988), raising copepodites in isolation provided an extra check on potential within-lineage 'contamination' from deposited spermatophores that may have occurred prior to paired mating experiments (e.g. Burris & Dam 2015). After sex determination, males and females were paired for within and between-lineage crosses and placed in 30 ml of fresh seawater at 7 ppt within sterile 35-mm-diameter well plates fitted with 7.5-cm-tall polycarbonate towers. In 'successful' matings (parents alive for ≥48 h and each sex represented), egg production, hatching rate, and nauplii survival were recorded every 24 h, at which time the media in each column was pipetted out, the well bottom cleaned of fecal matter and excess algae, and fresh *Rhodomonas* added. After 72 h, the adults were removed, rinsed in deionized water, and fixed in ethanol for genotyping. The columns were removed as well for easier counting of eggs and nauplii. Experiments were allowed to run for up to 5 d. All adults from 'successful' Set 2 crosses were validated with the RFLP assay to ensure that the correct lineage was represented by each parent (see Figs. S1 & S2 in the Supplement for lineage typing results for some crosses). As an additional validation of the RFLP test, and to further confirm the status of putatively pure F and S lineage cultures, we PCR amplified 12–16 individuals from each culture that were still alive at the end of all experiments in 2016 (cultures were continued for a few months after experiments ended), and performed both the *HaeIII* RFLP test and Sanger sequencing on the same amplicon, to verify RFLP assay performance (see Figs. S3 & S4 in the Supplement). Bidirectional sequencing for this test was performed at the Arizona State University DNA lab. In no cases did the RFLP test or analysis of raw sequences reveal contamination of either culture, and the 2 methods produced corresponding lineage results for all individuals tested (Figs. S3 & S4 in the Supplement). Sequences generated from the lineage validation are archived in Genbank (accession nos. MH376311–MH376338).

Virgin egg production

Virgin female egg production was examined as a control to assess the egg production and hatching success of virgin females in the absence of mates, to provide a comparison against egg production data from crosses. Approximately 10 CI–CIII copepodites were isolated in clear 60 ml plastic jars and followed until maturity using the same light, temperature, and feeding conditions as their parent cultures. Five of this group developed into females and were monitored subsequently. Once mature, the 5 females were placed in fresh jars for monitoring of egg production and maintained there for 10 d. Every 24 h, eggs were counted and removed to a 'group' jar, which held all the eggs produced by a given female, and which received the same daily examination and aeration as the jars with females. Females whose eggs hatched into nauplii were not considered virgin females, and were removed from the experiment (n = 1). Females not producing nauplii in their 'group' jars (n = 4) were kept under experimental conditions for up to 240 h for a full count of egg production.

Morphological and CHN analyses of lineages

Adult males and females examined for prosome length and CHN content were selected from each lineage-specific culture and imaged under a Leica S6D stereo dissecting microscope, on which was mounted a Canon EOS Rebel T1i fitted with a Martin Microscopes MM-SLR adapter at 4× magnification. All individuals were measured for length (prosome) using Image J 1.5 (Schneider et al. 2012) and after pictures were taken, subsets of these individuals were either placed into vials of ethanol for genotyping to confirm lineage as described previously (see *HaeIII* RFLP lineage-typing results in Figs. S1 & S2 in the Supplement), or were placed onto pre-combusted glass fiber filters for CHN analysis. Each filter for CHN analysis contained 8–12 individuals of the same sex and from the same lineage. Pooling was done to ensure enough material for a robust measurement signal. At the time of collection, there were enough females from each culture so that 3 filters from each lineage were used for CHN analysis; however, there were only enough males for one filter per lineage. Filters were folded in half, and dried at 50°C for at least 7 d, then kept in a desiccator until analysis on an Exeter Analytical (EAI) CE-440 Elemental Analyzer. Images of each individual were tracked so that we were able to calculate mean and standard deviation of the prosome lengths from each CHN filter and the individuals that were used for haplotype analysis. Mean prosome lengths from each filter were then regressed against the C and N weight measurements for that filter to compare the relationship between prosome length and weight for the different lineages.

Statistical analyses

All statistical analyses and graphics were done in R v. 3.3.0 (R Core Team 2016) using base statistical packages, 'ggplot2' (Wickham 2009) and 'PMCMR' (Pohlert 2014). Maps of sampling locations were made in Matlab (R2017a, MathWorks). Variation in egg production rate (EPR) among experiments and cross types was assessed with 1-way ANOVAs; however, non-parametric analyses (Kruskal-Wallis rank sum test and post-hoc Kruskal-Nemenyi tests; Pohlert 2014) were used to examine differences in hatching rate among cross types and experiment sets because the data failed assumptions of normality even after multiple transformations (e.g. arcsine square-root).

RESULTS

Crossing experiments

Of approximately 40 paired matings set up among *Acartia tonsa* adults from the 2 sets of experiments, 27 resulted in viable or 'successful' experiments, in which both parents were alive for at least 24 h after pairing and each sex was represented—data from crosses that lacked either of these characteristics were not analyzed. Of these 27 successful matings, 15 were within-lineage crosses (F×F or S×S), 12 were hybrid crosses (S×F or F×S), and all produced eggs (Table 1). Daily EPR was fairly similar among cross types, averaging 38.25, 39.25, and 31.48 for F×F, F×S, and S×S, respectively (Fig. 2). Within cross types, EPR was highly variable among females, and no significant differences in mean EPR were observed among cross types (ANOVA $p = 0.6854$) nor was there a significant difference in mean EPR between experiment sets (Set 1 vs. Set 2, ANOVA $p = 0.986$).

Hatching rate was highest for the within-lineage cross-types, averaging 0.52 and 0.46 for F×F (n = 8) and S×S (n = 7), respectively, while mean hatching rate was much lower for the between-lineage crosses (0.02, n = 12; Fig. 3). Only 1 out of the 12 between-

Table 1. Daily egg production rate (EPR) and hatching rate among cross types

Expt. ID	Cross	Eggs	EPR	Nauplii	Hatch rate (%)	Expt
1	F×F	25	25.00	15	0.6	Set 1[b]
2	F×F	23	23.00	12	0.52	Set 1
3	F×F	34	34.00	22	0.65	Set 1
4	F×F	26	26.00	16	0.62	Set 1
5	F×F	62	62.00	43	0.69	Set 1
6	F×F	54	18.00	24	0.44	Set 2
7	F×F	180	60.00	118	0.66	Set 2
8	F×F	174	58.00	0	0	Set 2
9	S×F[a]	18	18.00	0	0	Set 1
10	S×F	16	16.00	3	0.19	Set 1
11	S×F	42	42.00	0	0	Set 1
12	S×F	90	90.00	0	0	Set 1
13	S×F	58	58.00	0	0	Set 1
14	S×F	156	52.00	0	0	Set 2
15	S×F	58	19.33	0	0	Set 2
16	S×F	54	18.00	0	0	Set 2
17	S×F	85	28.33	0	0	Set 2
18	S×F	96	32.00	0	0	Set 2
19	S×F	140	46.67	0	0	Set 2
20	S×F	152	50.67	0	0	Set 2
21	S×S	30	30.00	21	0.7	Set 1
22	S×S	31	31.00	17	0.55	Set 1
23	S×S	23	23.00	16	0.7	Set 1
24	S×S	102	34.00	58	0.57	Set 2
25	S×S	24	24.00	0	0	Set 2
26	S×S	117	39.00	0	0	Set 2
27	S×S	166	55.33	115	0.69	Set 2

[a]All hybrid or between-lineage crosses are represented as F×S, though a mix of crosses in both directions (F male × S female or S male × F female) were performed.
[b]Set 1 experiments allowed ~24 h of egg production or mating time before removing parents; Set 2 experiments allowed 72 h before parents were removed.

lineage crosses produced any nauplii (3 nauplii; Table 1), though eggs were observed in all 12 crosses (Table 1). Non-parametric statistical analyses indicated that hatch rate varied significantly among cross types (Kruskal-Wallis ranked sum test, $p < 0.001$) and post-hoc comparisons (Kruskal-Nemenyi tests) showed that mean hatch rate in the between-lineage crosses was significantly different from that of the 2 within-lineage crosses ($p = 0.006$ for F×F vs. F×S, $p = 0.012$ for S×S vs. F×S) but hatch rate was not different between the F×F and S×S cross types ($p = 0.995$).

Virgin egg production

Virgin egg production in the absence of a mate was followed for 4 females over 10 d. As shown in Fig. 4, egg production began around Day 3 and continued through the 10 d of the experiment, with peak egg production between Day 5 and Day 8 and a decline thereafter. Total egg production ranged from 236 to 445 eggs (mean 339.74) or a mean production rate of ~34 eggs d^{-1}, similar to EPR estimates from the crossing experiments.

Morphological and CHN measurements

Morphological and chemical measurements of laboratory raised cultures of *A. tonsa* revealed substantial differences between the 2 lineages. Image analysis of 104 adult copepods showed that the mean size (prosome length) of copepods from the F lineage was significantly lower than the S lineage for both females (mean F vs. S, 0.757 vs. 0.877 mm; T-test $p < 0.0001$), and males (mean F vs. S, 0.674 vs. 0.772 mm, T-test $p < 0.0001$; Fig. 5). F lineage copepods were about 13–14 % smaller (males

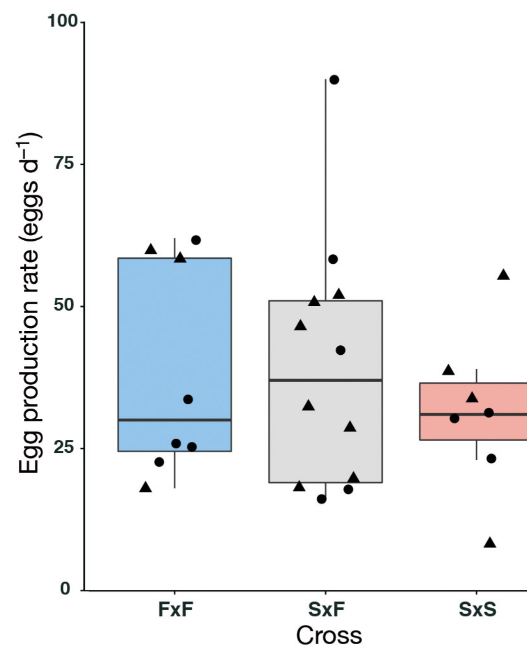

Fig. 2. Box and whisker plots for *Acartia tonsa* daily egg production rate (eggs per female per day) among cross types. Filled circles represent data points from females in Set 1; triangles represent females from Set 2 experiments. Thick lines within boxes represent the median egg production rate for a given cross type, lines bounding boxes below and above represent the 25 % and 75 % quartiles, respectively, and the lower and upper whiskers extend to the smallest/largest value less than 1.5 times the interquartile range from the box hinge

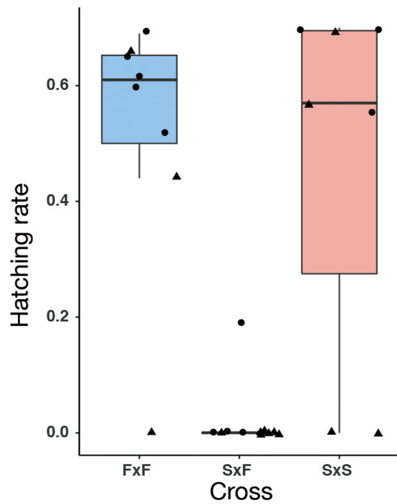

Fig. 3. Box and whisker plot of *Acartia tonsa* hatching rate among cross types for all mating experiments (n = 27). Thick lines within boxes represent the median hatching rate among matings for a given cross type, lines bounding boxes below and above represent the 25% and 75% quartiles, respectively, and the lower and upper whiskers extend to the smallest/largest value less than 1.5 times the interquartile range from the box hinge. Filled circles and triangles represent data points from Set 1 and Set 2, respectively

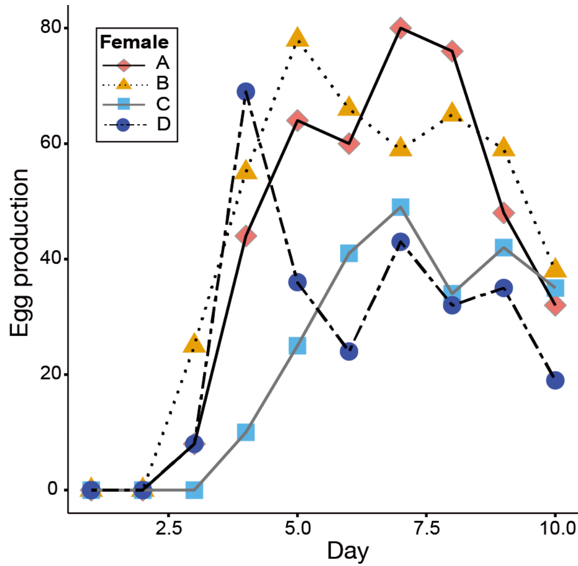

Fig. 4. Individual, daily egg production for 4 virgin *Acartia tonsa* females over 10 d. Each daily data point represents new egg production since the previous time point

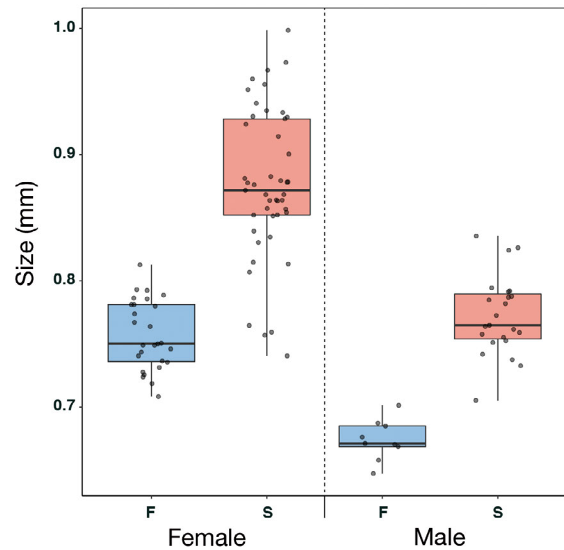

Fig. 5. Box and whisker plots of size (prosome length) measurements for male and female *Acartia tonsa*. F lineage in blue and S lineage in red—measurements for each individual copepod are plotted over the box and whisker plot. Thick lines within boxes represent the median length for a given lineage/sex, lines bounding boxes below and above represent the 25% and 75% quartiles, respectively. The lower and upper whiskers extend to the smallest/largest value less than 1.5 times the interquartile range from the box hinge

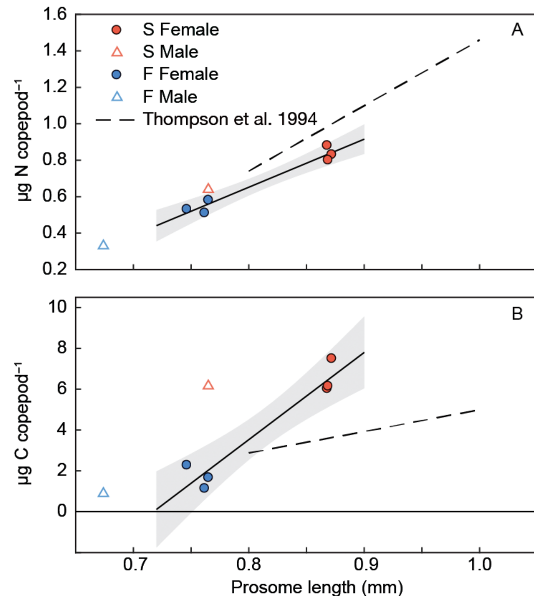

Fig. 6. Chemical composition data and regression results for (A) nitrogen and (B) carbon. Each point represents CHN analysis results from a pool of adult copepods (n = 8 or more), triangles represent males and circles represent females. F lineage points are blue and S lineage points are red. The black line and grey shaded area represent the fitted least squares regression line and 95% confidence interval of the slope for each elemental analysis (female data only; $r^2 = 0.95$ for nitrogen, $r^2 = 0.92$ for carbon). Dashed lines show the relationship between prosome length and elemental analysis from Thompson et al. (1994)

and females considered separately). Regression of carbon and nitrogen content per copepod against size (Fig. 6) produced strong linear relationships for females (µg C per copepod = 42.7252 × [prosome length in mm] – 30.6504; µg N per copepod = 2.6368 × [prosome length in mm] −1.4574) that were highly significant (p = 0.0007 and adjusted $r^2 = 0.95$ for nitrogen;

Table 2. Summary of length measurements and chemical composition (CHN) results. Prosome length measurements are means ± standard deviation for all individuals measured (n = 45 for S females, n = 27 for F females, n = 23 for S males, and n = 9 for F males). Carbon and nitrogen values represent means across copepod pool (n = 3 for females with at least 8 individuals per pool) ± standard deviation

	Prosome length (µm)	Carbon (µg)	Nitrogen (µg)	C:N
Females (S)	877 ± 59	6.55 ± 0.82	0.84 ± 0.04	7.85 ± 1.14
(F)	757 ± 27	1.69 ± 0.57	0.54 ± 0.03	3.11 ± 1.06
Males[a] (S)	772 ± 31	6.16	0.64	9.58
(F)	673 ± 16	0.89	0.33	2.67

[a]CHN estimates for males (F and S) were done on a single set of individuals (n = 9 and 11, respectively) so standard deviations were not calculated

$p = 0.002$ and adjusted $r^2 = 0.90$ for carbon). When comparing the slopes of the relationships between female prosome length and elemental content with previous work for *A. tonsa* in Narragansett Bay (Thompson et al. 1994), the slope for nitrogen appeared to be similar but the slope for carbon was not as steep. Individuals used in the present study had shorter prosome lengths than in that previous study (Thompson et al. 1994). Only 1 pooled filter of males was analyzed for each lineage, so regression analysis was not conducted. Substantial differences were also found between lineages in the per copepod carbon and nitrogen content (Table 2). For example, F lineage copepods had 74–85% less carbon per individual and 35–48% less nitrogen per individual (males and females) compared with the S lineage (Table 2). The ratio of carbon to nitrogen also varied between lineages, with the S lineage showing 2–3-fold higher C:N ratios in males and females (Table 2). Because both genotyping and CHN analyses are destructive to the organisms, we were not able to do both analyses on individuals. However, PCR genotyping of individuals from the respective cultures (N = 30 and 27 for F and S, respectively) confirmed expected lineages for copepods from those cultures (see Figs. S1 & S2 in the Supplement).

DISCUSSION

Reproductive isolation between F and S lineages

Based on the almost complete lack of nauplii hatching success in the 12 between-lineage (hybrid) crosses, reproductive isolation between the F and S lineages appears to be quite strong. A single hybrid cross did produce 3 nauplii (3/16 eggs hatched; Table 1), but given the results from the other 11 crosses, it is possible that these were not 'hybrid' offspring but instead were accidentally introduced from another within-lineage crossing vessel. *Acartia tonsa* nauplii are quite small (~70 µm) and thus could have been accidently entrained in a drop of water splashed between towers or vessels. In contrast, within-lineage crosses produced viable offspring (nauplii) in 12 of 15 crosses and these crosses were conducted concurrently with the between-lineage crosses, which indicates that experimental conditions were amenable to successful reproduction by both of the lineages. The lack of hatching success in the 3 within-lineage crosses may be due to a number of potential factors that were artefacts of our experimental setup, including poor condition of 1 or both adults, mis-sexing of one of the adults (e.g. 2 females present), or accidental separation of adults (caught in mesh or between tower and vessel walls) during the experiment, all of which could limit or prevent successful mating. However, *A. tonsa* reared in culture have shown variable hatching success that is dependent on a variety of factors, including temperature, salinity, prey quantity, and diet quality (Støttrup et al. 1986, Jónasdóttir 1994, Holste & Peck 2006), so some variability in hatching rate is perhaps not surprising. The finding of complete or nearly complete reproductive isolation between the F and S lineage is consistent with previous molecular data that showed deep divergences between the lineages (~18% at *COI* and 12% at ITS; Chen & Hare 2008). However, the degree of genetic divergence among cryptic species or lineages does not always correlate with reproductive isolation or offspring fitness, and few studies actually test reproductive success between lineages in lab-based experiments (e.g. Lee 2000, Edmands 2002). Lee (2000) found that neither divergence nor geographic distance were great predictors of reproductive success when crossing cryptic lineages of the freshwater copepod *Eurytemora affinis*. Our results show clearly that crosses between the 2 divergent lineages of *A. tonsa* are far less successful than within-lineage crosses, which provides strong evidence that some form of reproductive isolation is occurring between the 2 lineages.

Additional information about the biology and ecology of the 2 *A. tonsa* lineages may provide insight into the causes and development of the observed reproductive isolation. While the 2 lineages do overlap

in spatial distribution at lower (oligohaline) salinities in the Chesapeake Bay (~5–15 ppt; Table 4-1, Figs. 4-3 to 4-9 in Chen 2009), the F lineage is found primarily in salinities below 10 while the S lineage is found primarily above 12 ppt. Thus, subtle spatial or ecological separation ('micro-allopatry'; e.g. Smith 1965, Fitzpatrick et al. 2008) may have contributed to the development of reproductive isolation and subsequent deep genetic divergence (Edmands 2002). In addition, morphological and chemical composition data from this study show that the 2 lineages are actually quite morphologically distinct, differing significantly in size and in chemical composition (e.g. carbon per adult and C:N ratio). Differences in size among lineages had been suggested previously by Chen (2009), but differences in the chemical composition of the 2 lineages had not been considered—the CHN data are the first such data to show differences in chemical makeup between the lineages of *A. tonsa.* Taken together, the morphological observations from this study and Chen (2009) suggest that the 2 lineages are likely only 'pseudo-cryptic' (e.g. Lajus et al. 2015), as more detailed analyses of morphology and chemistry revealed clear differences among the S and F lineages.

The substantial (~15%) difference in size between lineages is particularly interesting because it could have very direct effects on mating behavior and compatibility between the lineages. For example, increased relative differences in size between F females and S males could result in reduced copulatory success, or perhaps prevent mating from initiating in the first place. Size differences may be indicative of additional morphological or behavioral divergence that would result in mating incompatibility, including pheromone production and hyrdomechanical cues (e.g. Bagøien & Kiørboe 2005, Goetze & Kiørboe 2008, Ceballos & Kiørboe 2010, 2011). Mate choice and mating behavior are important in copepods, and some evidence exists for sexual selection (Titelman et al. 2007, Ceballos & Kiørboe 2010, 2011, Ceballos et al. 2014); thus, subtle differences in behavior and chemical or acoustic cues between these lineages may prevent successful mating (Titelman et al. 2007, Goetze & Kiørboe 2008). While more study is needed of the specific reproductive characteristics that may differ among the F and S lineages, other studies have identified significant differences in vital rates (egg production, development) among divergent *A. tonsa* clades in the Eastern Atlantic (e.g. Drillet et al. 2008b), lending further support to the idea that there are prominent biological differences among cryptic *A. tonsa* lineages. Synthesizing the results of the data from this study and the ecological, biological, and genetic data published previously, there is strong evidence that these 2 lineages (and possibly other *A. tonsa* lineages) are reproductively isolated and likely represent separate species.

Nature and timing of reproductive barriers

Considering the strong indication that the F and S lineages do not interbreed and may represent separate species, it is instructive to consider the nature and timing in which these reproductive barriers might have arisen. Indeed, substantial effort in speciation research has been focused on identifying the reproductive barriers between diverging lineages or species, particularly whether they are pre-zygotic (e.g. pre-mating ecological isolation, mating incompatibilities, or gametic incompatibilities; e.g. Coyne & Orr 2004, Presgraves 2010, Kozak et al. 2012) or post-zygotic (e.g. hybrid breakdown or inviability, Dobzhansky-Muller interactions; Orr & Turelli 2001), which may provide some inference about the timing or extent of isolation. Prezygotic barriers are demonstrated to evolve first for some species (e.g. Coyne & Orr 1997, Bolnick & Near 2005, Stelkens et al. 2010), although the opposite has also been shown (e.g. Hendry et al. 2009, Kozak et al. 2012). Whether pre- or post-zygotic isolation develops more quickly may also depend on whether species are in sympatry versus allopatry, and in lab settings, its inference may be affected by the environmental conditions in which hybrids are reared (intrinsic vs. extrinsic post-zygotic isolation; Schluter 2001, Kozak et al. 2012).

For *A. tonsa* lineages in Chesapeake Bay, a lack of hatching success in 'hybrid' crosses in the lab suggests that post-zygotic isolation is either complete (embryos have zero fitness; Fig. 3) or it is not in play because pre-zygotic barriers are acting before fertilization. If pre-zygotic barriers are the major drivers of reproductive isolation here, there are still a number of potential mechanisms that could be acting at various points in the mating and reproductive process. Incompatibility isolation could occur during mating, in which behavior might differ between lineages such that spermatophores are never transferred (Titelman et al. 2007, Goetze & Kiørboe 2008). Reproductive morphology might also differ such that mating is attempted, but fertilization is impossible or mechanically compromised. Alternatively, mating and fertilization may be possible, but gametic incompatibility prevents egg and sperm from forming a viable zygote

(Orr & Turelli 2001, Edmands 2002, Coyne & Orr 2004). Because we did not track mating behavior during crosses, it is impossible to determine which prezygotic barrier might be most significant. Previous studies of mating behavior between congeneric versus more distant copepod species have shown a surprising amount of fluidity or lack of specificity in mating interactions; thus, significant genetic distance may not dictate divergent mating behaviors or mating incompatibilities. For example, Goetze & Kiørboe (2008) and Goetze (2008) showed that pheromone and hydromechanical pre-contact mating cues lack species specificity, suggesting that species recognition likely occurs during contact with chemical cues or is dictated by morphological differences. *A. tonsa* use hydromechanical cues, not pheromones, to locate mates (Bagøien & Kiørboe 2005), so it is possible that these cues may differ among lineages and if sufficiently divergent, would prevent the initiation of mating. We did not attempt to monitor behavior during matings, but we did image females under the microscope for the last 7 crosses during mating (at the end of the 72 h period with the male), and we observed that the females from all 4 hybrid crosses lacked attached spermatophores, while 67% of females from within-lineage cultures did have attached spermatophores (C. Fitzgerald unpubl.), which is consistent with Burris & Dam (2015), who found that approximately 80% of *A. tonsa* females observed in Long Island Sound had attached spermatophores at any given time. Our observations are not fully quantitative, but they do suggest a potential mechanism of pre-zygotic isolation acting at the mating or premating stage (behavioral or morphological) that prevented attachment or transfer of spermatophores. Further analysis of mating behavior will be required to provide a more comprehensive assessment of the mechanisms of pre-zygotic isolation using high-frequency videography or other detailed behavioral assessment.

The timing of divergence between the 2 lineages remains unknown, but our crossing results and previous molecular data suggest that isolation is probably not recent. Previous molecular analyses indicate that the 2 *COI* haplotype groups are ~13–17% divergent, and a molecular concordance test between *COI* and the ITS2 locus demonstrated complimentary, reciprocal monophyly for both genes (Chen & Hare 2008), which is indicative of long-term isolation. Assuming an average rate of divergence of 2.4% per million yr for animal mitochondrial DNA (e.g. Rand 1994, Edmands 2002), one can estimate that the 2 lineages may have been separated for as long as ~6–7 million yr, though this estimate is likely to be only very roughly correct (Coyne & Orr 1997, Sasa et al. 1998). Substantial variation in evolutionary rates exist among taxa and among genes, and *COI* may not be the ideal marker to estimate divergence, as it does not always resolve known or expected species within some copepod taxonomic groups (e.g. Blanco-Bercial et al. 2014). Coalescent modeling of divergence timing across various demographic scenarios may provide a better approach to test hypotheses about the timing and nature of lineage divergence; however, caution must be exercised in the interpretation of isolation with migration models (Hey & Nielsen 2007, Hey 2010), when inferring speciation timing (e.g. Gaggiotti 2011, Strasburg & Rieseberg 2011). Given that the 2 *A. tonsa* lineages in Chesapeake Bay overlap in time and space in lower-salinity waters, it might be tempting to refer to them as sympatric species, but distinguishing between sympatric speciation (speciation with gene flow) or allopatric speciation followed by secondary contact will require more analysis and may prove difficult (e.g. Bolnick & Fitzpatrick 2007, Fitzpatrick et al. 2008, Gaggiotti 2011). Overall, more detailed genomic analyses and observation of mating behavior will be needed to elucidate the nature and timing of reproductive isolation between the lineages of *A. tonsa* in Chesapeake Bay.

Potential ecological and food-web implications of cryptic divergence

The findings of substantial differences in size, chemical composition, and biology between the 2 lineages of *A. tonsa* have serious implications for their potential value as prey items and thus for trophic dynamics in estuarine ecosystems. In Chesapeake Bay, *A. tonsa* is a critical food source for a number of important fisheries species, including larval striped bass and bay anchovy (e.g. Houde & Secor 2009, Martino & Houde 2010, Shideler & Houde 2014), and a change in the size or the amount of energy obtained from consumption of *A. tonsa* may be important for early larval survival of these fishes. The 13–14% smaller size and roughly 70% reduction in carbon content of F lineage males and females may translate to significantly less energy acquired per copepod consumed, and thus a shift in the relative abundance of the 2 lineages could have a significant effect on larval fish energetics and recruitment (e.g. Cowan et al. 1993, Mazur et al. 2007). While the S lineage is found across a wider range of salinities (~5–30 ppt) and is thus present throughout much of

Chesapeake Bay, it is relatively less common in the more fresh and brackish environments (<7 ppt; where F is common) that serve as nursery sites of larval striped bass (North & Houde 2003, Martino & Houde 2010, Shideler & Houde 2014). Varying environmental or climatic factors that affect the amount of freshwater in the bay may influence the relative frequency of the 2 lineages and thus the quality of copepod prey available. For example, in wet years with increased precipitation and greater freshwater flow, the bay-wide proportion of *A. tonsa* that are F lineage may increase, resulting in smaller adult individuals and lower prey quality for fish (less food per copepod consumed). Annual variation in precipitation or freshwater flow conditions in Chesapeake Bay have previously been associated with changing plankton community composition, which correlated to changes in indices of production or recruitment of striped bass and other fish (e.g Kimmel et al. 2009, 2012, Wood & Austin 2009), but this may have been driven more by shifts in the extent and location of the estuarine turbidity maximum (e.g. North & Houde 2003) rather than simply the change of salinity and its effect on *A. tonsa* distribution and prey quality. Of course, other prey items are also significant in the diets of striped bass larvae (e.g. *Eurytemora carolleeae* and *Bosmina* spp; North & Houde 2003, Houde & Secor 2009, Martino & Houde 2010) and prey size or carbon content in *A. tonsa* may be a weak predictor of 'quality' for particular fish species. Further, it is possible that additional biological or chemical properties that differ between *A. tonsa* lineages, such as lipid composition, may be more important. Analyses of potential differences in amount and composition of lipids between the lineages are ongoing, but our current data show that the C:N ratio varies between the lineages, which relates to lipid content in other copepod species (e.g. Campbell et al. 2001). More information about how divergent lineages of *A. tonsa* respond to environmental change and differ in their quality as prey items may be important for understanding early survival of fish species in Chesapeake Bay and other estuaries on the US east coast, and will be the subject of upcoming studies.

CONCLUSIONS

Laboratory crossing experiments between 2 cryptic, divergent lineages of the copepod *Acartia tonsa* in Chesapeake Bay revealed strong, potentially complete reproductive isolation that may be pre-zygotic. This result supports previous molecular data and analyses that suggested long-term reproductive isolation between the lineages. Additional detailed analyses of the morphology and chemical composition of the 2 lineages revealed substantial differences in length (14% smaller for F lineage) and carbon content (~70% lower in F lineage) that could significantly impact the quality of each lineage as prey items. Based on our crossing data, the morphological analyses, and synthesizing previous molecular and biological data on *A. tonsa*, we suggest that these cryptic lineages may be different species. However, additional molecular analyses and perhaps coalescent modeling will be needed to understand the timing and nature of divergence and speciation. Finally, the results of this study should motivate future work to understand the ecological effects of cryptic divergence in zooplankton in coastal ecosystems, specifically how future climate shifts and environmental stress may alter the abundances of cryptic lineages and their value as prey items, which has serious implications for trophic dynamics and fisheries production.

Acknowledgements. This research was funded by the National Science Foundation (NSF) Division of Ocean Science and Maryland Sea Grant via the Research Experiences for Undergraduate Program (OCE-1262374) and the University of Maryland Center for Environmental Sciences.

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Editorial responsibility: Sigrun Jónasdóttir, Charlottenlund, Denmark

Submitted: September 18, 2017; Accepted: March 16, 2018
Proofs received from author(s): May 25, 2018