Ann. Limnol. Int. J. Lim. 50 (2014) 37 47
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DOI: 10.1051/limn/2013069

Available online at:
www.limnology journal.org

Dispersal of *Corbicula fluminea*: factors influencing the invasive clam's drifting behavior

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Abstract – *Corbicula fluminea*, is one of the most successful invasive species in fresh and brackish waters. Dispersal is one of the most determinant steps in the invasive process, and the full understanding of the mechanisms involved in this step is critical for adequate pest management both in the wild and in industries affected by this species' biofouling activity. A mucous drogue line produced by mucocytes packed along the inner demibranchs of the clams' gills seem to play an important role in assisting drifting and hence dispersal. Two Asian clam populations geographically separated (one in the USA and the other in Portugal), investigated at different times of the year, were reported to differ in terms of mucous drogue line production and floating. In this study, genetics and seasonality effects were hypothesized to explain the difference between the populations. To test these hypotheses, the two populations were genetically compared, and the Portuguese one was followed for 14 months to record the animals' mucous drogue line production and flotation capabilities and locate the population reproductive periods. Our results signal a possible scenario of micro evolution with consequences on the production of the clams' mucilaginous drogue line. Although some authors advocate a link between mucous threads formation and reproduction events, such a relationship was not observed in this study. By contributing to the understanding of a physiological trait of the Asian clam that is important for dispersal, this study may be of practical relevance for pest monitoring and control.

Key words: Asian clam / mucous drogue line / flotation behavior / life cycle traits / invasion

Introduction

Invasive species are a major worldwide threat due to the serious ecological and economic impacts they are responsible for (Pimentel *et al.*, 2005; Barinova *et al.*, 2010). Lakes, estuaries and rivers are particularly prone to invasion (Ricciardi and MacIsaac, 2000) because of the large number and variety of transport vectors and anthropogenic disturbance agents affecting these systems (Cohen and Carlton, 1998). Dispersal into a new habitat is a determinant stage in the invasion process (Davis, 2010), and hence the full understanding of the mechanisms involved in this stage is a key asset to manage the pests.

Among the most successful aquatic invaders is the freshwater bivalve *Corbicula fluminea* (Müller, 1774), commonly known as the Asian clam (DAISIE, 2008). Several life-cycle traits contribute to its success as an invader, including high growth rates, short life spans, high fecundity rates (up to 600–700 juveniles.day ^{1}; Aldridge and McMahon, 1978) and hermaphroditism sometimes associated with self-fertilization (Britton and Morton, 1982; Kraemer and Galloway, 1986). These traits combined with increased waterborne traffic resulted in the massive expansion of *C. fluminea* from its native distribution range in Southeast Asia to vast regions in Europe (Araujo *et al.*, 1993) and North (Phelps, 1994) and South America (Ituarte, 1994) over the last century.

A particular life-cycle trait of this species that has been considered to contribute to its dispersal abilities is the production of a mucilaginous drogue line by modified cells (mucocytes) packed along the inner demibranchs of the ctenidia of juvenile and young adult clams. This drogue line was proven to assist clam flotation (in clams up to 14 mm shell length) in response to water currents, thus promoting drifting into new locations and favoring the species dispersal (Prezant and Chalermwat, 1984).

Although mucilaginous drogue line production may play an important role in the species' dispersal, being

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potentially relevant from the pest management point of view little is known about this physiological feature. In a previous study, Rosa *et al.* (2011) reported marginal industrial biofouling effects and mild invasion by *C. fluminea* in Portugal even though the species entered the country more than 30 years ago (Mouthon, 1981). As a possible explanation for this unexpected observation, it was hypothesized that environmental conditions could constrain the production of the mucilaginous drogue line thus affecting the species' dispersal. In a subsequent study (Rosa *et al.*, 2012), conducted in early spring (April), a USA population of *C. fluminea* was examined and temperature was shown to influence this trait. When the authors investigated the mucilaginous drogue line production and drifting behavior of a Portuguese Asian clam population later in summer (between June and August), implementing similar methodologies, neither mucocytes nor any flotation-like event were observed. Here a follow-up study is reported, with two major hypotheses being investigated to clarify the inconsistency observed among the two clam populations.

One of the hypotheses tested was that the two populations could represent different haplotypes of *C. fluminea* (Hypothesis 1). Given that the *Corbicula* genus shows high genetic variability and significant phenotypic plasticity has been attributed to the species (Glaubrecht *et al.*, 2003; Park and Kim, 2003; Sousa *et al.*, 2007; Pigneur *et al.*, 2011), different haplotypes could result in physiological differences regarding drogue line production and hence drifting abilities. To test this hypothesis, the American and Portuguese clam populations were genetically characterized and compared. In parallel, the hypothesis that mucilaginous drogue line production could vary seasonally, which would explain the different behaviors observed in spring and summer, was also investigated (Hypothesis 2). Some authors (*e.g.*, Morton, 1977; Byrne *et al.*, 2000) suggested, although providing no clear supporting evidence, that the mucous threads in *C. fluminea* have an additional role in nourishing the embryos, also developing in the inner demibranchs, and/or in assisting the release of juveniles out of the gills. This being the case, an annual variation of mucous production, concomitant with the animals' reproductive cycle, could be expected. To assess this hypothesis, the Portuguese clam population was followed for 14 months as a study model. The animals' flotation behavior and the production of mucilaginous threads by ctenidial mucocytes were analyzed throughout the test period. The population's reproductive period(s), potentially connected with mucous production, were also located by following the seasonal changes in the clams' body condition (as dry tissue weight) and the population dynamics, complementing with morphological examination of the clams' gills to identify the presence of offspring.

Because *C. fluminea* is a serious aquatic invader, with both ecological and industrial impacts, it is important to enlarge the body of knowledge on the species' biology. The study of the Asian clam dispersal mechanisms, namely by characterizing possible seasonal patterns and the life-cycle traits linked to the process, may contribute to the design of more effective monitoring strategies and improved control methods for the nuisance.

Materials and Methods

Assessing Hypothesis 1: Genetic typing of the two *C. fluminea* populations

The genetic characterization of both the American (see Rosa *et al.*, 2012 for details on this population) and the Portuguese populations (see below for details on the collection site) was done through restriction fragment length polymorphism (RFLP) analysis of the mitochondrial cytochrome *c* oxidase subunit I gene (mtCOI) plus sequencing of the gene (Renard *et al.*, 2000). Twenty clams (shell length above 22 mm) were collected from each population. The animals' valves were opened by gently forcing them with a blunt needle, and each individual was placed in a vial filled with absolute ethanol PA at 4 °C in order to preserve DNA integrity. Total DNA was extracted from approximately 30 mg of each individual using the E.Z.N.A.® Mollusc Isolation Kit as indicated by the manufacturer. A 710 bp fragment of the mtCOI gene was amplified by polymerase chain reaction (PCR) using the primers designed by Folmer *et al.* (1994): LCOI490 (5'-GGTCAACAAATCATAAAGATATTG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATC-3'). A negative control (no DNA template) was used in the reaction. Amplification of 15 ng DNA occurred in a total volume of 25 µL and the reaction mixture also included 1 µM of each primer, 0.2 mM of each dNTP, 1 mM of $MgCl_2$, 0.025 U.µL^{-1} adjusted to 1 U.sample^{-1} of Taq DNA Polymerase (Fermentas, Lithuania) plus the recommended buffer. The PCR protocol consisted in an initial denaturation step of 60 s at 94 °C, 35 annealing/elongation cycles of 60 s at 94 °C, 60 s at 4 0 °C and 90 s at 75 °C, and a final elongation step at 72 °C for 5 min. The PCR product was then digested by the restriction enzyme Sac I (Takara, China): 5 µL of the PCR product with 5 U of the restriction enzyme plus the corresponding buffer as recommended by the enzyme manufacturer were incubated overnight at 37 °C; 5 U of restriction enzyme were further added to prolong digestion for 3 h; the reaction was terminated by adding 1 µL of loading buffer to each mixture. An agarose gel (1.5 % (w/v)) electrophoresis was run in order to confirm whether all individuals could be identified as *C. fluminea* (one band of 710 bp or two bands of 200 and 500 bp; Renard *et al.*, 2000). In order to further find the haplotype(s) present in the samples, the PCR product was purified (ExoSAP-IT®; Affymetrix, USA) and sequenced in a certified laboratory according to ISO 9001:2008, using the oligo LCOI490. The obtained sequences were compared with sequence data from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) using BLAST homology search. Similar sequences were aligned with the clams' sequences using CLUSTALW2

(EMBL-EBI) and the differences between the sequence of nucleotides were then assessed.

Assessing Hypothesis 2: Seasonal variation of the drogue line production

Study site and clam collection details

C. fluminea individuals were collected in Casal de São Tomé, Mira, Portugal (N40°25'06.90"/W8°44'13.18"), from a sandy muddy shallow creek, which is connected to other brooks, forming a network of small canals. Clams were collected monthly from October 2011 to December 2012 and twice a month between May and September 2012, the latter being expected to cover the breeding period. Two different sampling strategies were employed depending on the subsequent analysis. Qualitative sampling was used to obtain organisms for histological studies and analysis of drifting behavior as detailed below. Clams were collected by using a shovel to drag sediment into a bag with 1 mm mesh size, used to roughly sieve the sample. Clams with shell length in the range 9–14 mm, corresponding to mature animals that were already proven to be able to produce a mucilaginous drogue line (Prezant and Chalermwat, 1984; Ituarte, 1985), were then selected. Quantitative sampling was used to address the seasonal variation of the clams' body condition and obtain cohort frequencies (see below for details). Clams were collected with a Van Veen grab ($15 \times 32 \times 19.5$ cm) from three sampling points along a transect established in the creek. The sediment collected was dragged into a 1-mm mesh size bag to roughly sieve the sample, which was then transferred to a 20-L bucket filled with *ca.* 15 L of field water for transportation to the laboratory.

Temperature, pH, conductivity and dissolved oxygen contents were measured *in situ* with a multiparameter field probe (WTW-Multi3430). Flow speed was estimated by examining the traveling time of a floating plastic cylinder. Water samples (*ca.* 4 L) were collected and vacuum filtered (GF/C 1.5-µm pore filters) for further determination of turbidity through the calculation of the absorption coefficient (m^{-1}; Brower *et al.*, 1997). The filtration residue was used to quantify the total suspended solids ($mg.L^{-1}$; APHA, 1995) and photosynthetic pigments ($\mu g.L^{-1}$ Chl *a*; Lorenzen, 1967). Sediment was also collected to quantify the loss-on-ignition organic matter content (% (w/w); ASTM, 2000).

Analysis of the seasonal changes in clams' flotation behavior and drogue line production

Clams' flotation behavior was observed *in situ* and in the laboratory. In the field, ten clams (shell length in the range 9–14 mm) were randomly drawn from the qualitative sample previously collected (see above for details) and placed into a plastic container with dimensions $35 \times 25 \times 6$ cm. The clams were subjected to the field water flow by keeping the container fully flooded for 4–5 min and their flotation activity (if any) was recorded. In addition, as the clams were observed to be actively siphoning, some drops of 5% (w/v) toluidine blue were placed near the siphons to assess whether a mucous drogue line was being produced. The clams were then transported to the laboratory in plastic containers filled with field water, which was then gradually replaced by dechlorinated municipal water for acclimation purposes. The sample was kept under continuous aeration in a culture room at constant temperature (20 ± 2 °C) and photoperiod (16 h^L:8 h^D). After 24 h, the clams were transferred into a 15-L aquarium, containing 14 L of dechlorinated municipal water, and placed inside a submerged crystallizing dish to continue the acclimation period for another 24 h. An externally driven recirculation and filtration system was set in the aquarium to ensure the water quality. Following the approach suggested by Prezant and Chalermwat (1984) and Rosa *et al.* (2012), the clams were submitted to a gentle water flow and their flotation behavior (if any) was recorded over a 30-min period.

Microscopic inspection of the ctenidial mucocytes distributed in the inner demibranchs of clams sampled qualitatively was performed to get additional insight into the mucilaginous drogue line production and support the interpretation of the flotation data. Ten clams (shell length ranging from 9 to 14 mm) were transported in plastic containers filled with field water to the laboratory, where they were immediately opened through gentle forcing between valves with a blunt needle. Their soft tissues (whole body) were fixed in Zenker's fluid overnight. The fixed samples were treated as detailed by Rosa *et al.* (2012). Briefly, they were washed and dehydrated, and then the tissues were carefully embedded in paraffin wax (Paraffin mp 56–58 °C, Merck KGaA), so that anteroposterior sections could be made in the organisms, corresponding to longitudinal sections of the demibranchs. The blocks were sectioned at 5–7 µm cut thickness. The sections were stained with sodium borate buffered aqueous toluidine blue. The first eight sequential sections of the demibranchs were selected for microscopic examination to assess the presence of mucous cells (Olympus CKX41 inverted microscope).

Identification of the population's reproductive period(s)

Data on both the seasonal variation of the clam's body condition and the population dynamics, complemented by the morphological examination of the clams' gills, were employed to locate the population's reproductive periods.

The clams were sorted from the quantitative samples brought from the field (see above for details). The total clam numbers were counted and the animals' shell lengths were measured with a digital caliper to the nearest 0.1 mm. Subsamples of 30 clams covering the whole samples' size range were taken to establish allometric relationships between biomass (dry weight of the soft tissues obtained by drying to constant mass at 60–80 °C) and shell length. The model $DTW = a \cdot L^b$, where DTW and L represent

clams' dry tissue weight (mg) and shell length (mm), respectively, was fitted to the data by plotting ln DTW against ln *L*. Data showing a Cook's distance greater than one were eliminated since they can be considered influential (Quinn and Keough, 2002). Whenever the probability distributions of ln DTW at each ln *L* were normal, a Model I regression was applied (ordinary least-squares). In cases where normality was not observed (November and December 2011, and January and March 2012), a Model II regression was applied (ranged major axis regression) (Legendre and Legendre, 1998). Coefficients of determination (r^2) were determined in order to estimate the proportion of the total variation in ln DTW that was explained by the allometric relationship with ln *L* (Quinn and Keough, 2002). Analysis of covariance (ANCOVA) followed by the Tukey's multiple comparison test was applied to compare the slopes of the regression models obtained over the study period using ln *L* as a covariate (Quinn and Keough, 2002). Regression lines determined by the Model II regression approach were excluded from this analysis as the extension of this model to ANCOVA cannot be applied (Quinn and Keough 2002).

Ten clams (shell length in the range 9–14 mm) were randomly collected from each qualitative sample (see above for details on sampling) to examine the presence of offspring in the gills. This evaluation was used to infer *C. fluminea* maturation state and to morphologically identify the onset of the reproductive period(s). After collection, the clams' valves were immediately opened by forcing them with a blunt dissection needle and the whole organism was preserved in 96% (v/v) ethanol. At the laboratory, extemporaneous microscopic slides were prepared by gently smashing and smearing the gills into the slide with the cover slip. Whole homogenized alcohol samples were also observed to ensure that any offspring released during preservation could be tracked. The slides were observed under an inverted light microscope (Olympus CKX41) and the presence/absence of offspring was recorded.

Results

Assessing Hypothesis 1: Genetic typing of the two *C. fluminea* populations

Genetic typing of the Portuguese and the American clam populations revealed that there is no clear distinction between them as both exhibit the same haplotype (*COI* haplotype I; GenBank: AF269090-3).

Assessing Hypothesis 2: Seasonal variation of the drogue line production

Analysis of the seasonal changes in clams' flotation behavior and drogue line production

No clam drifting was observed *in situ* or in the laboratory throughout the entire study period. Also, no toluidine blue staining was observed in the field, indicating that the mucous drogue line was not being produced by the clams observed. The clams, both *in situ* and in the laboratory, distended their exhalant siphon and foot when exposed to the water flow, but only small movements with no clear lifting off the substratum for further drifting into the water column were observed. Microscopic inspection of the clams' inner demibranchs generally revealed no ctenidial mucocytes (Fig. 1(a)). The histological

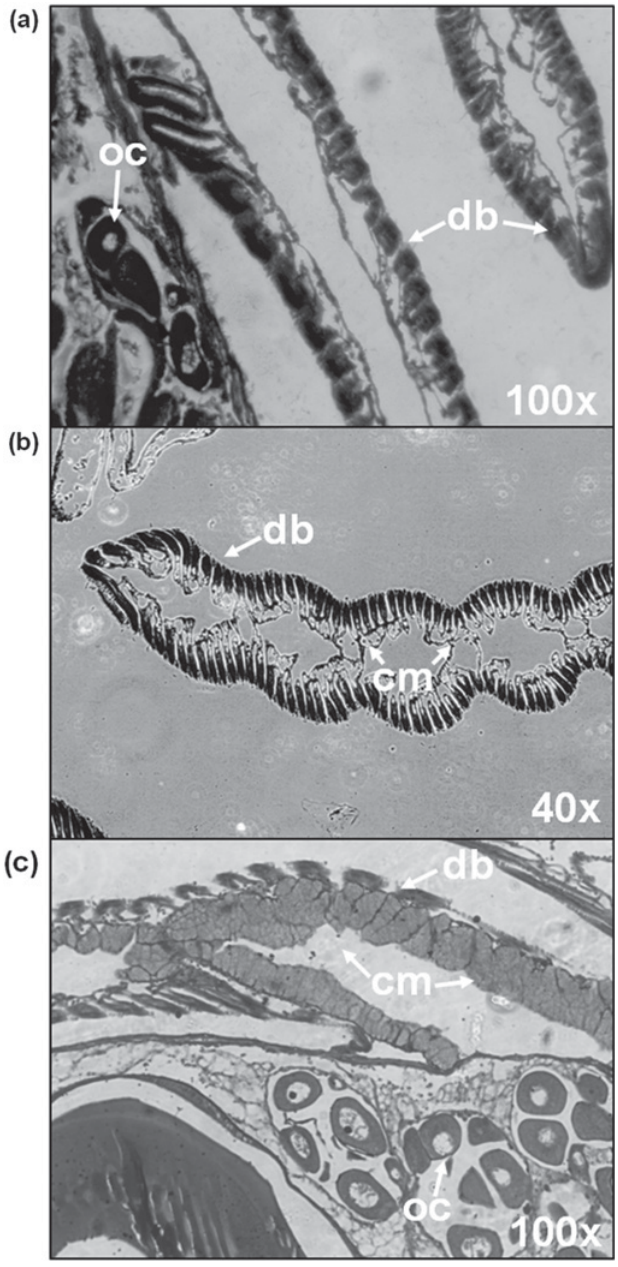

Fig. 1. Overview of the visceral mass of *C. fluminea* focusing oocytes (oc) and inner demibranchs (db), (a) without ctenidial mucocytes (cm), (b) with incipient mucocytes and (c) with abundant mucocytes. Panels (a) and (b) refer to the Portuguese clam population while panel (c) refers to the American *C. fluminea* population (collected from Sommerset, NJ, USA) analyzed by Rosa *et al.* (2012).

Table 1. Estimated parameters of the allometric model $DTW = a \cdot L^b$ (DTW in mg and L in mm) and respective coefficients of determination (r^2). The number of allometric equations statistically differing from that found for each sampling date (ANCOVA followed by the Tukey's *post hoc* test; $P < 0.05$) is also presented; allometric models based on Model II regression approach were excluded from this analysis (see Material and Methods section for details), being denoted by ' '.

Sampling date	a	b	r^2	No. of distinct regressions
01 Oct 2011	111	2.66	0.943	5
03 Nov 2011	162	2.51	0.917	
09 Dec 2011	153	2.52	0.814	
04 Jan 2012	165	2.49	0.888	
06 Feb 2012	354	2.27	0.821	7
05 Mar 2012	117	2.62	0.827	
10 Apr 2012	773	2.04	0.856	10
02 May 2012	2117	1.65	0.840	6
16 May 2012	1422	1.79	0.865	7
04 Jun 2012	388	2.18	0.925	2
15 Jun 2012	257	2.28	0.918	6
02 Jul 2012	330	2.23	0.914	3
18 Jul 2012	424	2.16	0.907	3
31 Jul 2012	132	2.49	0.906	9
13 Aug 2012	159	2.53	0.931	4
03 Sep 2012	218	2.33	0.903	7
17 Sep 2012	314	2.19	0.918	8
01 Oct 2012	324	2.26	0.851	4
07 Nov 2012	420	2.11	0.908	5
05 Dec 2012	728	1.94	0.801	4

screening showed mucocytes in the demibranchs of individuals sampled in the first two months of the study (October and November 2011). Overall, mucocytes were observed in 15 out of the 20 individuals sampled in those months. However, such mucocytes seemed to stand in an incipient state as clearly shown in Figure 1(b) by comparison with the "swollen" cells shown in Figure 1(c).

Identification of the population's reproductive period(s)

The general allometric equation relating dry tissue weight and shell length was successfully fitted to the several data sets obtained throughout the study period ($P < 0.05$ in all cases; Table 1 and Fig. 2). Such gradients were significantly affected by the collection date (ANCOVA: $F = 12.46$; d.f. = 15, 469; $P < 0.05$), with each allometric model differing from at least two other equations characterizing distinct data sets (*post-hoc* Tukey's test; $P < 0.05$). Figure 3, obtained from the allometric models (Table 1), shows the seasonal change of dry tissue weight of two hypothetical standard organisms: a juvenile with 8 mm shell length and an adult with 25 mm shell length. The model juvenile is characterized by generally constant physiological condition throughout the sampling period although its dry tissue weight undergoes a slight increase during spring, followed by a decrease in early summer, which are compatible with incipient reproduction. As for the model adult clam, the change of the allometric models translates into a clear and more pronounced annual pattern of body condition: the dry tissue weight of such an individual increases from late winter over spring and suffers a marked drop in late

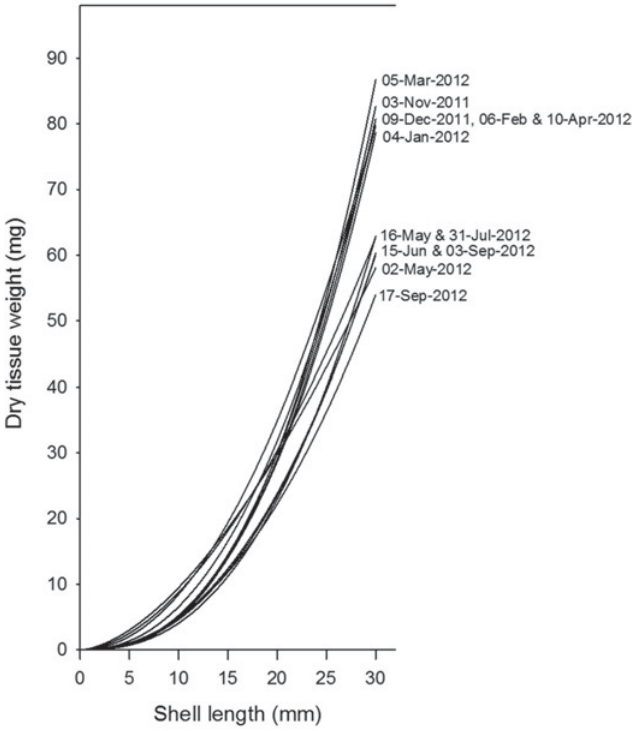

Fig. 2. Allometric models characterizing *Corbicula fluminea* body condition on selected occasions between October 2011 and December 2012. The curves were drawn using the parameters presented in Table 1. For clarity purposes, only models statistically differing from at least six other equations (ANCOVA followed by the *post hoc* Tukey's test; $P < 0.05$) and models found through Model II regression were represented in the figure.

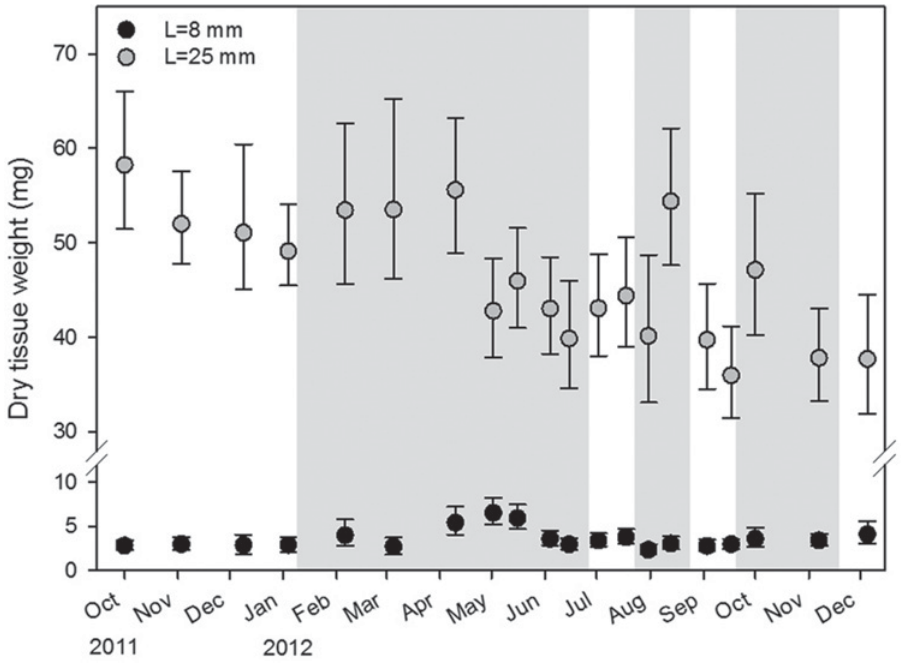

Fig. 3. Seasonal variation of dry tissue weight of two model clams with 8 and 25 mm shell length during the sampling period. The underlying allometric models are presented in Table 1 and Figure 2. Error bars represent 95% confidence intervals. Shadowed areas represent the periods where morphological examination showed offspring in the parents' gills.

spring; over summer the body condition keeps generally lower than in spring, fluctuating in a series of peaks, with tendency to stabilize in late autumn. This seasonal pattern of body condition is consistent with a primary release of offspring in late spring, followed by multiple spawning events over summer until mid-autumn. The difference, in absolute terms, of the body condition of clams collected in equivalent periods of 2011 and 2012 (October–December) is worth noticing, with the animals collected in 2012 having significantly lower dry tissue weight (but following a similar trend in both the periods). Such a difference can be related to different environmental conditions in the two periods; the lower water temperature in October to December 2012 as compared with the equivalent period in 2011 (see supplementary Fig. S1 available online at the address: http://www.limnology-journal.org) should have played its role in this context.

Morphological examination showed that the presence of offspring in clams' inner demibranchs scattered across the study period as shown by the shadowed areas in Figure 3, signaling a more continuous-like breeding behavior for the population, consistent with the multiple spawns mentioned above. Offspring were found in parents' gills even in November and December 2012.

Figure 4 shows the clam population density and mean shell length in the study site over the sampling period. Overall, clam density was (mean $\pm$ SD) 3247 ± 1122 clams.m ^{2}, with a maximum peak of over 6000 clams.m ^{2} recorded in August 2012 and minimum densities of < 1500 clams.m ^{2} reached in April, May and December 2012 (Fig. 4). The highest values of mean shell length were recorded in April and May 2012 (mean $\pm$ SD: 17.59 ± 3.80 and 17.40 ± 3.57 mm, respectively), while on average the clams were the smallest in November 2012 with a mean shell length of (mean $\pm$ SD) 14.00 ± 2.74 mm (Fig. 4). In June and in the beginning of August, clam's mean shell length was also low as compared with the rest of the year (mean $\pm$ SD of 14.44 ± 3.60 mm and 14.12 ± 3.34 mm, respectively) (Fig. 4). Figure 5, where size frequency distributions are presented, further documents the dynamics of the population structure over the study period. Young adults with shell length between 11.5 and 17.5 mm tended to be the most abundant size class, dominating the population size structure. The fraction of juveniles (shell length up to 10 mm; Ituarte 1985; Cataldo and Boltovskoy 1998; Mouthon 2001a) in the population was always low compared with that of the adults. Juvenile fraction noticeably increased over June 2012, decreasing in the beginning of the following month. Then, it peaked again in late July 2012, and juveniles kept being found until mid-September 2012. A final increase of the juvenile fraction was observed in November 2012. Such variation of the population size structure is consistent with multiple spawning events occurring from late spring through summer until mid-autumn. The recruitment periods identified in summer and autumn (Fig. 5) consistently occurred 1–2 months after drops in the clams' body condition indicative of spawning (Figs. 2 and 3). An earlier, less significant recruitment event seemed to happen in March 2012 (Fig. 5), 5 months after the previous spawn in October 2011 (Figs. 2 and 3), indicating that the progeny released in autumn takes longer to grow over winter as compared with the individuals born in the warm months.

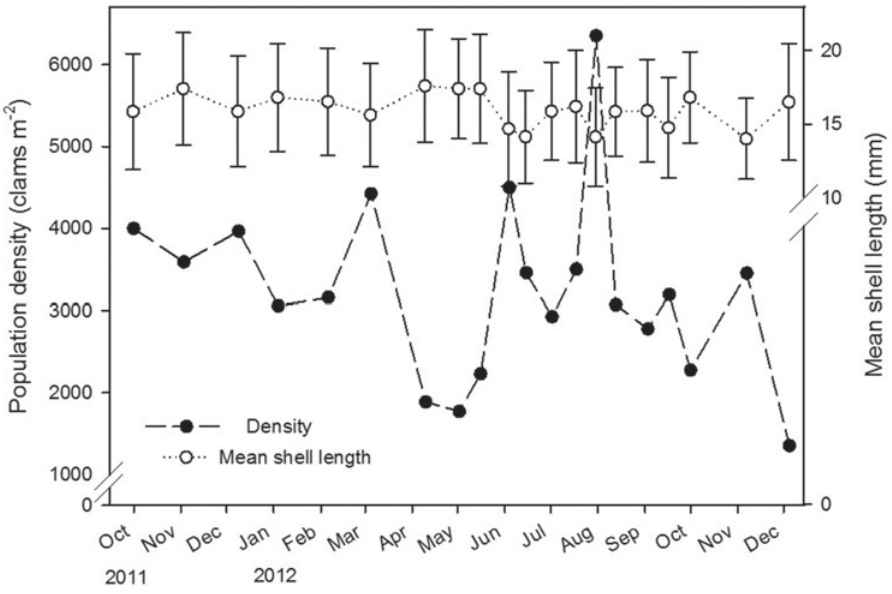

Fig. 4. Variation of the population density (left *yy*' axis) and mean shell length (right *yy*' axis) during the sampling period. Error bars represent SD.

Discussion

Understanding the process through which the Asian clam produces a mucilaginous drogue line that promotes its flotation and drifting into new areas may contribute to more effective pest management. As this process was investigated in two *C. fluminea* populations geographically separated (one in Sommerset, NJ, USA and the other in Portugal), at different times of the year, unexpected differences were observed – mucous threads and floating responses, reported for the American clam population (Rosa *et al.*, 2012) were completely absent among the organisms drawn from the Portuguese population. It was hypothesized that such differences could be due to genetic differences between the two populations and/or seasonal effects, and these two hypotheses were hence investigated in this study.

The taxonomy of the *Corbicula* genus is generally confused and yet unsolved because of the recognized high phenotypic variation in shell morphology, color and ornamentation, contrasting reproductive strategies and ecological differences (Glaubrecht *et al.*, 2003; Park and Kim, 2003; Sousa *et al.*, 2007; Pigneur *et al.*, 2011). It was shown here that there is no clear genetic distinction between the American and the Portuguese *Corbicula* populations, both exhibiting the same haplotype – haplotype I as described by Renard *et al.* (2000) in Europe, which corresponds to *Corbicula* sp. (Form A) described by Lee *et al.* (2009) in the USA and *C. leana* described by Park and Kim (2003) in Asia. It should be noted that androgenesis is a common feature of the *Corbicula* genus (Komaru and Kawagishi, 1998; Byrne *et al.*, 2000; Ishibashi *et al.*, 2003), which compromises analysis relying only on mitochondrial sequences as conducted in this study *i.e.*, distinct nuclear lineages can be grouped in the same mitochondrial cluster (Park and Kim, 2003; Hedtke *et al.*, 2008; Pigneur *et al.*, 2011). Further genetic studies, combining nuclear and mitochondrial data along with detailed morphological examination, should thus be carried out for a definite genetic comparison of the two populations of interest.

Some authors (Morton, 1977; Williams and McMahon, 1987; Byrne *et al.*, 2000) suggested that the mucous produced in *C. fluminea* demibranchs play an important role in reproduction, namely by assisting the nourishment of the developing embryos and the release of offspring out of the parents' gills. The mucilaginous drogue line production and associated drifting behavior could thus be expected to vary seasonally concomitantly with the species reproductive cycle.

In this study, data on the animals' body condition seasonal pattern, gills' morphological examination and population size structure dynamics were integrated to locate the reproductive period(s) of the Portuguese clam population. These data consistently indicated that breeding occurred from late spring until mid-autumn, with multiple spawns in a more continuous-like reproductive pattern over this period. Major recruitment events in June, August–September and November were identified based on the population dynamics analysis. Reproduction of *C. fluminea* is known to vary greatly with location (Table 2), which suggests a strong influence of environmental and genetic factors in life-cycle-related events. Although one recruitment episode (from early summer to early autumn) or two episodes (one in spring/early summer and other in late summer/early autumn) are most commonly reported for Asian clam populations, the pattern observed in this study is in agreement with many other works that also show recruitment occurring in mid/late-Autumn (Table 2).

Despite the reproductive patterns observed in the clam population, no mucocyte cells could generally be found in

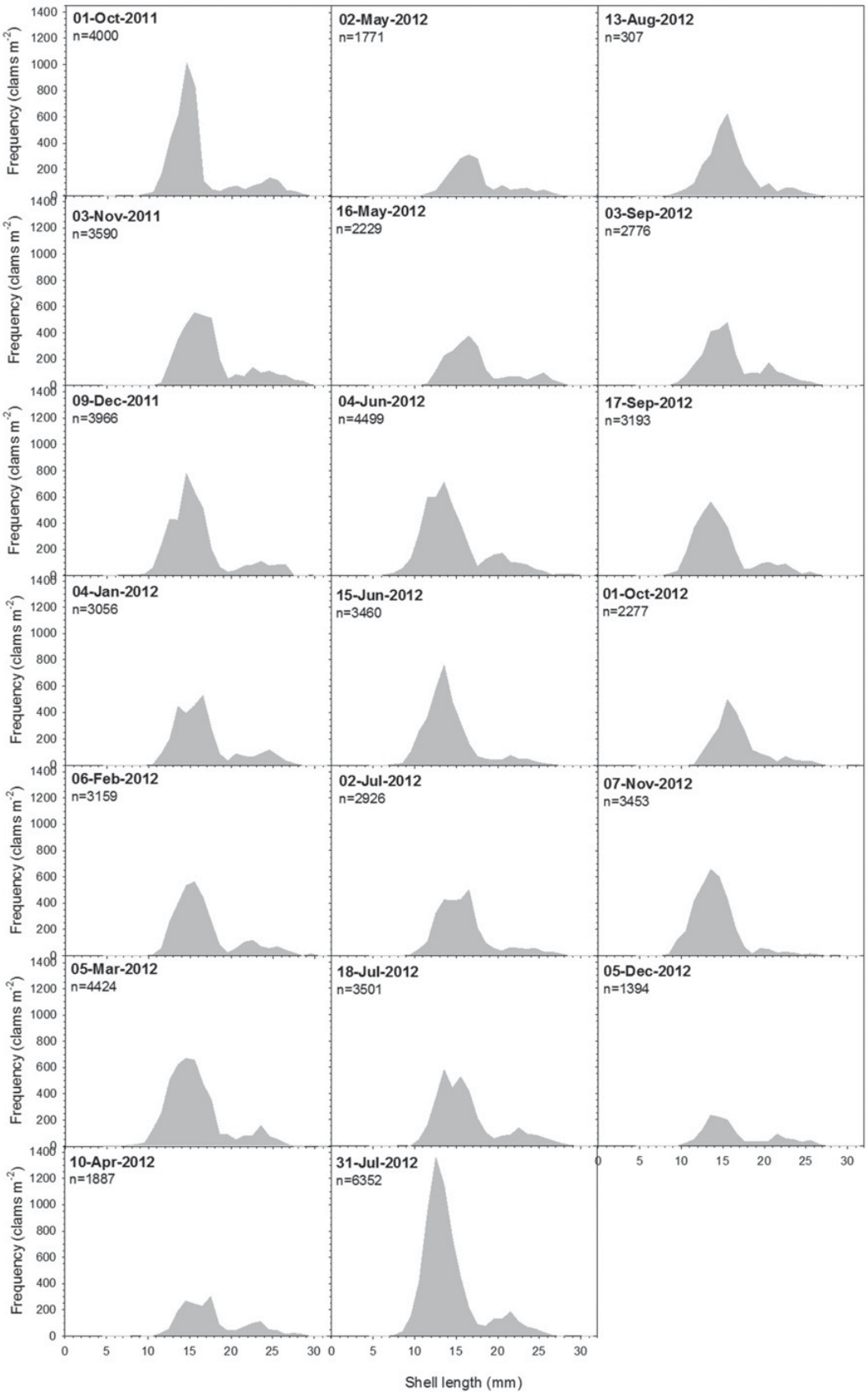

Fig. 5. Size frequency distribution of the clam population during the sampling period (October 2011 to December 2012). *n* represents the total clam density (clams.m^{-2}).

Table 2. Summary of shell length, clam density and recruitment events for several *C. fluminea* populations. The study site location, sampling date and water temperature range are also shown. This summary result from a thorough literature search and only studies reporting data on at least four of the columns were considered.

Study	Study site location	Sampling date	Water temperature range (°C)	Shell length range (mm)	Clam density range (clams.m ^{2})	Recruitment events
Aldridge and McMahon (1978)	Lake Arlington, USA	Sep 1974 Jan 1976	12.2 32.2	0.2 40	17.7 94.6	Apr Jul & Aug Dec
Ituarte (1985)	Punta Atalaya, Argentina	Nov 1982 Apr 1985	~ 11 27	~ 3 ~ 30	36 1132	September
McMahon and Williams (1986)	Trinity River, USA	Sep 1980 Dec 1982	4.8 29	2.6 ~ 45	305 16198	Mar Jul & Aug Nov
Hornbach (1992)	Mechums River, USA	Oct 1982 Oct 1983	0 ~ 26	0.4 19	173 1495	July
French and Schloesser (1996)	St. Clair River, USA	1988 1990	0.5 12.5	1.8 5.3	18 187	
Cataldo and Boltovskoy (1998)	Paraná River Delta, Argentina	Oct 1995 Oct 1996	10 29	0.2 33	379 2609	Oct Nov
Rajagopal *et al.* (2000)	Lek River, Netherlands	Aug 1991 Jan 1993	~ 2 ~ 24			May/Jun & Sep
Mouthon (2001a)	Saone River, France	Sep 1986 Dec 1999	0.5 27.2	1.5 ~ 29	~ 120 934	Jun/Jul & Aug/Sep
Mouthon (2001b)	Rhône River, France	Sep 1996 Dec 1999	3 23.5	~ 0.5 ~ 29.3	~ 300 5266	Jul Sep
Morgan *et al.* (2003)	Connecticut River, USA	Aug 1993 Nov 1994	1.7 30.6		52 114	Jun Sep
Mouthon and Parghentanian (2004)	Loire River, France	Dec 2001 May 2003	[Ice] 25	0.5 ~ 34	88 < 4000	Jan Feb & May Oct
Sousa *et al.* (2006)	Lima River, Portugal	Aug 2004 & Aug 2005	20.1 22.9	13.0 51.6	60	
Schmidlin and Baur (2007)	Alrhine River, Switzerland	Mar 2003 Oct 2003	7 24	1 24	83 339	Jun Jul
Sousa *et al.* (2008)	Minho River, Portugal	Jan 2005 Aug 2006	6.7 23.1	1.85 41.83	92 2152	
Franco *et al.* (2012)	Mondego Estuary, Portugal	Dec 2007 Dec 2008	10.1 25.4	0.92 37	419	
Present study	Casal de São Tomé, Portugal	Oct 2011 Dec 2012	11.8 20.5	6 32	1353 6352	Jun Sep & Nov

the tested animals' gills (in the cases where mucocytes were observed, and the cells were in an incipient state) over the 14-month study. This result does not definitely contradict the link between mucous production and reproduction-related events – in populations where mucous is actually produced, mature parents' developing embryos and offspring release from the parents' gills may benefit from it as defended by some authors (Morton, 1977; Williams and McMahon, 1987; Byrne *et al.*, 2000). However, the reported data prove that mucous production is not a necessary condition for successful clam reproduction to occur. Previous studies on mucocytes showed that the mucous threads are produced by juvenile and small adult clams (up to 14 mm shell length), and, together with other behavioral mechanisms, assist the drift of the clams through flotation in response to water currents (Prezant and Chalermwat, 1984; Rosa *et al.*, 2012). The absence of mucocytes cells in the inner demibranchs of the test clams is thus consistent with the absence of drifting, both in the field and in the laboratory, throughout the sampling period.

As the American and the Portuguese clam populations were proven to be genetically similar and season-driven effects were ruled out, the question why the two populations have different mucous production and drifting capabilities remains unanswered. It can be speculated that, while corresponding to the same species, the two populations are different due to a high rate of genotypic and/or phenotypic plasticity. It is possible that the colonization of different habitats triggered microevolutive events towards local adaptation (Ashley *et al.*, 2003; Sousa *et al.*, 2007), which may explain the inhibition of the drogue line production and drifting behavior in the Portuguese clam population. Further studies are now necessary to assess this hypothesis.

Overall, this study confirms that mucocytes development is directly related to Asian clam drifting behavior and indicates that microevolutionary events may condition these processes. It thus contributes to the understanding of a physiological trait of the species that is important for dispersal and hence relevant from the pest management point of view. As already discussed, the absence of conspicuous drogue line production and drifting behavior is likely to constrain the species dispersal; and, in fact, although reported in Portugal since the early 1980s (Mouthon, 1981), *C. fluminea* has not yet invaded all the country's watersheds, contrary to the USA where faster expansion seems to have occurred (Counts, 1986). Also, the impacts in Portuguese freshwater-dependent industries are relatively mild (Rosa *et al.*, 2011) opposing to serious damages reported by the American facilities (Pimentel *et al.*, 2005). This suggests that decreased/absent mucocyte activity and hence slower clam's dispersal, may add to extended lag-phase periods or massive die-offs events (as discussed in Rosa *et al.*, 2011) to explain the reduced economic impacts observed in the country. The findings of the present study may represent an additional asset to refine the Asian clam pest management in the sense that the inhibition of the mucous drogue line production can be faced as a new target in the development of improved control strategies.

Acknowledgements. The authors are grateful to João Simões, Carolina Madeira and Artur Alves for their advice regarding

genetic analysis, as well as to Robert Prezant for his help in interpreting histological results. Bruno Castro, Henrique Queiroga and Alexandre Caseiro provided important guidance on the analysis of the population data. We would also like to thank all colleagues who helped in the field work as well as Rebecca Shell for collecting the clams from the USA population used in the genetic analysis. Inês Correia Rosa and Joana Luisa Pereira are recipients of individual scholarships by the Portuguese Foundation for Science and Technology (FCT) (Ph.D. scholarship SFRH/BD/33395/2008 and Post Doctoral scholarship SFRH/BPD/44733/2008, respectively). This study was supported by the European Regional Development Fund EDRF, through the Operational Competitiveness Programme COMPETE, and by national funds through FCT under the scope of the project PTDC/AAC AMB/113515/2009,CICECO PEst C/CTM/LA0011/2013 is acknowledged.

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