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POPULATION ECOLOGY - ORIGINAL RESEARCH

Tracking larvae with molecular markers reveals high relatedness and early seasonal recruitment success in a partially spawning marine bivalve

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Abstract The partial synchronized spawning strategy adopted by some marine invertebrate broadcast-spawners can lead to the production of many distinct pools of larvae within a single reproductive cycle. Following the fate of these larval groups from birth to settlement with molecular markers might shed light on mechanisms regulating their population recruitment. Larvae and recruits of *Mya arenaria*, a partially spawning marine bivalve, were monitored and collected over 13 consecutive weeks during an entire reproductive cycle. Each sampled individual ($n = 218$) was sorted according to size (early veligers, late veligers, post-larval recruits) and genotyped at seven microsatellite loci for comparisons among samples and with adult reference samples ($n = 270$). While traditional differentiation statistics (e.g., pairwise Δ_{ST}, allelic richness) suggested the absence of sweepstakes reproductive success, the level of relatedness found within and among larvae and recruit samples suggested otherwise. Four samples out of ten were observed to have higher within-sample relatedness values than randomly expected, including the very first group of early veligers produced in the season (E1) and the last group of post-larvae who survived recruitment (P10). E1 individuals were also found to be more related than randomly expected to individuals of more than 80 % of all other samples including the last surviving recruits (P8 and P10). These results suggest that the first larvae produced in the season were the most successful to survive recruitment. Results also show direct evidence for larval retention and demonstrate for the first time larval and post-larval kin aggregation in a marine bivalve.

Keywords Sweepstakes reproductive success · *Mya arenaria* · Larval retention · Broadcast-spawners · Kin aggregation · Microsatellites

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Introduction

Understanding the mechanisms regulating population recruitment of broadcast-spawning marine benthic invertebrate populations has posed a difficult challenge to ecologists for many years, mostly associated with the difficulty of working with very small larvae. As more technological advances are made, one of the most promising avenues to tackle these ecological issues lies in the use of molecular tools (Selkoe and Toonen 2006; Hedgecock 2010). Some studies have shown the presence of fine-scale genetic structure within large-scale homogeneous clusters, mostly owing to temporal genetic heterogeneity among recruits of different year-classes (e.g., Johnson and Black 1982; David et al. 1997; Varela et al. 2009). These findings are

consistent with the basic principles of a well-documented ecological theory called sweepstakes reproductive success (SRS) (see review of Hedgecock and Pudovkin 2011). The SRS theory hypothesizes that long-lived marine animals producing large numbers of offspring have extremely large variances in individual reproductive success leading to the appearance of "chaotic" patterns of genetic patchiness among annual cohorts of recruits. Successful individuals surviving recruitment are thus suggested to issue from a small fraction of the total adult breeding population that randomly or actively matches reproduction with optimal oceanographic conditions favoring gonad development, survival of pelagic larvae and proper larval development from birth to recruitment (Hedgecock and Pudovkin 2011).

In a recent literature review, Hedgecock and Pudovkin (2011) suggested SRS to be the main factor driving the genetic patterns of recruitment for highly fecund and long-lived marine organisms. However, several studies involving marine bivalves came to contradictory conclusions by showing no or very low genetic differentiation between annual cohorts of recruits originating from the same location (e.g., Cassista and Hart 2007; Taris et al. 2009; St-Onge et al. 2013). Although these contradictory results may be caused by several factors, notable differences in spawning and gamete-release strategies adopted among benthic marine invertebrates may be playing an important role.

Strategies of gamete release can usually be assessed by monitoring gonad masses or by histology (e.g., Himmelman et al. 2008; Cardoso et al. 2009; Enriquez-Diaz et al. 2009). For example a massive synchronized spawning (MSS) event corresponds to when breeders in a population synchronously release entire gonadic contents. Many authors have argued that MSS should be the most commonly encountered strategy in externally fertilizing and broadcast-spawning marine invertebrates to increase the probability of fertilization success hindered by rapid diffusion rates and limited life span of gametes in the water column (e.g., Levitan and Petersen 1995; Yund 2000; Himmelman et al. 2008; Mercier and Hamel 2010). However, such obstacles to fertilization can be overcome if individuals spawn in dense aggregated patches or in very large populations (e.g., Levitan and Young 1995; Marshall 2002; Gaudette et al. 2006), thus resulting in a wide array of gamete-release strategies that depend less on gamete concentration and that vary among species, locations, habitats and environmental conditions (e.g., Lauzon-Guay and Scheibling 2007; Cardoso et al. 2009; Simon and Levitan 2011).

One of these derived strategies is the partial synchronized spawning (PSS) strategy, corresponding to the synchronous release of only a fraction of the gonadic contents. PSS events are repeated throughout the reproductive cycle until gonads are completely depleted, redeveloped or resorbed (e.g., Barber et al. 2005; Cardoso et al. 2009). Some species are mostly characterized as PSS strategists (e.g., Cledon et al. 2004; Barber et al. 2005; Cardoso et al. 2009) which suggests that in some cases, PSS should represent a valid strategy to ensure recruitment success. Other temperate species can adopt both PSS and MSS strategies depending on the age of the individuals or environmental conditions, as observed in giant scallops *Placopecten magellanicus* (Langton et al. 1987; Cyr et al. 2007), bay scallops *Argopecten irradians irradians* (Bricelj and Krause 1992), blue mussels *Mytilus edulis* (Myrand et al. 2000) or Pacific giant oysters *Crassostrea gigas* (Royer et al. 2008; Enriquez-Diaz et al. 2009; Cardoso et al. 2013).

In a previous study, no evidence for temporal genetic structure or SRS was found among different year classes in two separate and genetically distinct softshell clam (*Mya arenaria*) populations (St-Onge et al. 2013), a partially spawning species of marine bivalve with a long planktotrophic development phase (e.g., Cardoso et al. 2009; Shanks 2009). These results lead to the hypothesis that PSS might be responsible for limiting the genetic differentiation of annual recruits. Using molecular tools such as highly polymorphic microsatellite markers would allow us to study the temporal variation of genetic diversity and relatedness as well as the existence of genetic patchiness throughout larval development until the final stages of the recruitment process. This approach would also allow a better understanding of the temporal components that are likely to modulate recruitment success. Up until now, these kinds of studies were scarce, mostly due to difficulties associated with developing polymorphic genetic markers, especially for bivalves, and with extracting DNA from single microscopic larvae. But recent technological advances have now rendered this kind of approach much more feasible (Larsen et al. 2005; Zhan et al. 2008; Barker et al. 2011).

In this study, we therefore postulate that breeding populations of partial broadcast-spawners such as the endobenthic marine bivalve *M. arenaria* can produce genetically similar recruits to that of the surrounding adult population by producing several distinct pools of larvae spawned over a long period of time that cancel out potential effects of SRS. The specific objectives will be to use a population genetic approach to assess whether we can observe the presence of genetic patchiness and evidence of SRS among all stages of the *M. arenaria* life cycle. This will primarily be done by testing for the two following null hypotheses: no genetic differentiation among samples of larvae, post-larval recruits and adults; and no difference in average relatedness between individuals present within and among each of the collected samples comparatively to all other individuals in the data set. Finally, the presence of SRS will also be assessed by investigating temporal patterns of genetic diversity from one larval development

Table 1 Information regarding 13 consecutive weeks of *Mya arenaria* sampling in Bouctouche Bay (Southern Gulf of St Lawrence, Canada; 046°31′N, 064°41′W)

Week post-FSE	Date (2010)	V_{WS} (L)	T (°C)	S (ppt)	Early veligers			Late veligers			Post-larvae		Adults
					n_E	n_{PI}	n_{IA}	n_E	n_{PI}	n_{IA}	n_E	n_{IA}	n_{Adults}
−2	2–8 May	50	13.5	24.0	0	–	–	0	–	–	–	–	2
−1	9–15 May	50	11.9	24.9	0	–	–	0	–	–	–	–	2
FSE (0)	16–22 May	144	14.3	24.9	5	1	0	0	–	–	–	–	1
1	23–29 May	216	15.1	24.4	58	47	25	1	0	–	–	–	1
2	30 May–5 June	216	15.1	25.6	38	35	24	2	0	–	0	–	6
3	6–12 June	144	14.1	24.8	7	0	–	32	30	11	33	27	6
4	13–19 June	216	18.9	24.1	2	0	–	75	63	36	–	–	4
5	20–26 June	–[a]	22.3	23.7	9	8	8	3	0	–	30	27	4
6	27 June–3 July	216	17.7	24.2	2	0	–	38	34	20	–	–	2
7	4–10 July	216	28.5	25.3	0	–	–	0	–	–	–	–	6
8	11–17 July	–	24.3	25.2	–	–	–	–	–	–	27	23	5
9	18–24 July	–	–	–	–	–	–	–	–	–	–	–	6
10	25–31 July	–	24.2	24.6	–	–	–	–	–	–	24	17	6
Total					121	91	57	151	127	67	114	94	51

FSE First spawning event (19 May 2010), V_{WS} volume of water sampled, T water temperature, S salinity, n_E number of specimens extracted for DNA, n_{PI} number of specimens positively identified as *M. Arenaria*, n_{IA} number of specimens included in genetic analyses, n_{Adults} number of adult specimens collected for the calculation of the gonado-somatic index (GSI)

[a] Data not available due to pump malfunction

stage to another from spawning to adulthood, sibship relations within samples and temporal variance in the number of breeders (N_b) responsible for producing individuals in samples.

Materials and methods

Sample characteristics

This study was carried out in the summer 2010 in the Bouctouche Bay (Southern Gulf of St Lawrence, Canada; 046°31′N, 064°41′W) sheltered by a 12-km-long sand dune acting as a potential barrier to larval dispersal (Online Resource 1). The sampling site was located near the protrusion of the sand dune from the mainland at the edge of a 100-m-wide intertidal sandflat with a slope of 0.24 % where resides a large and extensively harvested natural settlement of *M. arenaria* individuals (LeBlanc and Miron 2006). This site is part of the larger Southern Gulf of St Lawrence (SGSL) *M. arenaria* genetic cluster described in St-Onge et al. (2013).

Spawning of *M. arenaria* was monitored over a period of 13 consecutive weeks from 8 May 2010 to 27 July 2010 (Table 1) by dissecting adult specimens and by calculating the gonado-somatic index (GSI) (Online Resource 2) as in Roseberry et al. (1991). Larvae were sampled weekly from the water column during flood tide with a 12-V bilge pump and a series of meshed sieves (74, 200 and 405 μm) for a period of 10 consecutive weeks from 8 May 2010 to 7 July 2010 (Table 1). The first spawning event (FSE) was confirmed by both GSI (Online Resource 2) and the arrival of bivalve veligers on 19 May 2010 (Table 1), hereafter referred to as the FSE. Every week, larval samples of differing developmental stages were collected based on a threshold of 160 μm, chosen arbitrarily to separate larvae into two distinct groups: early/newly spawned larvae (<160 μm of shell length), and late/settlement-ready larvae (>160 μm of shell length). This choice of threshold was based on the observed shell lengths of *M. arenaria* larvae in the SGSL, with D-shaped/post-D-shaped larvae measuring between 90 and 140 μm and mature pediveliger larvae (i.e., completely formed and actively searching foot) measuring between 180 and 210 μm (P. St-Onge, personal observation). Since *M. arenaria* pediveligers were first observed in larval samples 3 weeks post-FSE (6 June 2010), post-larval recruit sampling was initiated on 11 June 2010 and repeated at 5, 8 and 10 weeks post-FSE (Table 1). See Online Resource 3 for a more detailed account of the exact field sampling procedures.

The detailed descriptions of methods used (1) for extracting DNA of larval and post-larval recruits, (2) for genetically identifying veligers as true *M. arenaria* individuals using a single nested multiplex polymerase chain reaction assay (Larsen et al. 2005), and (3) for genotyping individuals at seven microsatellite loci (*Mar1*, *Mar3*,

Mar4, *Mar5*, *Mar6*, *Mar7* and *Mar8*) (Barker et al. 2011) are all provided in Online Resource 4. In summary, DNA was extracted from 386 larval and post-larval recruits (see n_E in Table 1). Species genetic identification was performed on all 272 sampled veligers (n_E) while genotyping was carried out on a total of 218 positively identified *M. arenaria* veligers (n_{PI}) and 114 post-larval recruits (Table 1).

Individuals genotyped at two or more loci were kept for all following genetic analyses (see total percent of loci covered in Table 2). The final analyzed data set (see n_{IA} in Table 1) was composed from 12 temporal samples taken at four distinct larval development stages of *M. arenaria*: three early veliger samples collected at 1, 2 and 5 weeks post-FSE (respectively E1, E2 and E5); three late veliger samples collected at 3, 4 and 6 weeks post-FSE (respectively L3, L4 and L6); four post-larval recruit samples collected at 3, 5, 8 and 10 weeks post-FSE (respectively P3, P5, P8 and P10); and two adult reference samples analyzed in a prior study (St-Onge et al. 2013), i.e., one representing the main sampling site of Bouctouche (A) and another one representing the entire SGSL genetic cluster. Sample size and mean shell length of each sample are presented in Table 2 and Online Resource 5, respectively. See Online Resource 4 for more details on the methods used for the genetic characterization of samples.

Statistical analyses

The temporal variation of genetic diversity throughout larval development of *M. arenaria* was investigated using several statistical approaches, all of which are fully detailed in Online Resource 6. In summary, global and pairwise Δ_{ST} (Weir and Cockerham 1984) as well as the actual differentiation estimator (D_{EST}) (Jost 2008) were first used to measure sample differentiation. Gene diversity statistics such as the mean within-locus allelic richness ($_{MWL}$AR), Nei's (1978) observed heterozygosity across loci (H_O) and non-biased expected heterozygosity across loci ($_{NB}H_E$) were used to measure genetic diversity throughout larval development relative to adult reference samples.

The relatedness coefficient (R_{xy}) calculated between two individuals x and y represents the proportion of homologous alleles identical by descent and commonly shared by both individuals as a result of their level of kinship (Falconer and Mackay 1996). Although a number of relatedness estimators using multi-locus genotypic data have been developed (Lynch 1988; Queller and Goodnight 1989; Li et al. 1993; Ritland 1996; Lynch and Ritland 1999; Wang 2002, 2007; Milligan 2003), none of them can be considered as universally superior to others with overall performance rather modulated by the type of markers used, the actual relatedness in the studied population and the mating characteristics of the studied species (Van de Casteele et al. 2001; Wang 2007, 2011).

The first step carried out in COANCESTRY version 1.0.1.2 (Wang 2011) was therefore to simulate data by using a file of known allelic frequencies present in the SGSL genetic cluster (St-Onge et al. 2013). In total, 600 fictitious pairs of individuals (dyads) with known levels of kinship (Falconer and Mackay 1996) susceptible to being encountered in the *M. arenaria* population under study were created, i.e., 100 of each full-sibling (true $R_{xy} = 0.500$), half-sibling (0.250), double-first-cousin (0.250), first-cousin (0.125), second-cousin (0.013) and unrelated dyads (0.000). True R_{xy} values were then compared with R_{xy} estimates obtained from seven relatedness estimators by calculating for each estimator both the bias and the residual mean-squared error (RMSE) as described in Wang (2007) (Table 3). Values from all moment estimators were truncated from 0 to 1 to allow proper comparisons with maximum-likelihood estimators.

Results showed that the triadic maximum-likelihood method (TrioML) (Wang 2007) gave the most consistent estimates through all possible levels of kinship, generating the lowest bias and RMSE values for four out of six levels of kinship (Table 3). Although the Ritland (1996) estimator generated the lowest bias and RMSE values for both unrelated and second-cousin dyads, it severely overestimated relatedness for higher related dyads such as half-sibs and full-sibs (Table 3). Therefore, TrioML (Wang 2007) was chosen to estimate relatedness within the empirical data set. Relatedness estimates differed substantially from one estimator to another (Online Resource 7). TrioML estimates were highly correlated (0.961) with DyadML estimates (Milligan 2003) but only moderately correlated with all other five estimators (0.471–0.715). TrioML individual pairwise relatedness coefficients ($_{IP}R_{xy}$) were thus calculated between all possible pairs of individuals (dyads) as implemented in COANCESTRY version 1.0.1.2 (Wang 2011). With 253 individuals, these amounted to a total of 31,878 $_{IP}R_{xy}$ values ($253 \times 252 \times 0.5$).

To assess if individuals within and among each sample were, on average, more related to each other than randomly selected individuals in the data set, the observed difference in mean individual pairwise relatedness ($\Delta_{MIP}R_{xy}$) was calculated between a first group of dyads issued from the tested combination of samples ($n = x$) and a second group containing all remaining dyads ($n = 31{,}878 - x$). For each comparison, the null distribution of $\Delta_{MIP}R_{xy}$ was assessed following 1,000 random dyad reshufflings that took into account the original sample size and missing data at each locus. Individuals were combined both within and among samples and were considered more related to each other

Table 2 Sample-specific genetic characteristics calculated over seven microsatellite loci for ten temporal samples of larval and post-larval recruits of *M. arenaria* collected in 2010 in Bouctouche Bay (New Brunswick, Canada; 046°31′N, 064°41′W) and two reference adult samples [main sampling site of Bouctouche (*A*) and the Southern Gulf of St Lawrence (*SGSL*)]

Sample[a]	Week post-FSE	Date	n	% LC	$_{T}n_{A}$	$_{MWL}AR \pm SE$ ($n = 4$)	$_{T}n_{PA}$	H_O	$_{NB}H_E$	F_{IS}	n_{FS}	$_{M}P_{FS}$	n_{HS}	% Sibship	N_{bEST} (95 % CI)
E1	1	25 May 2010	25	69.1	65	4.83 ± 0.58	5	0.73	0.80	0.10	5	0.52	49	18.0	20 (11–39)
E2	2	31 May 2010	24	76.2	78	5.06 ± 0.45	7	0.72	0.82	0.13	1	1.00	31	11.2	33 (19–65)
E5	5	24 June 2010	8	85.7	40	4.64 ± 0.51	1	0.71	0.81	0.14	0	–	6	21.4	19 (7–402)
L3	3	6 June 2010	11	81.8	44	4.26 ± 0.42	2	0.66	0.73	0.09	0	–	13	23.6	17 (8–47)
L4	4	14 June 2010	36	84.9	87	4.80 ± 0.55	1	0.73	0.79	0.08	0	–	78	12.4	32 (19–56)
L6	6	29 June 2010	20	76.4	59	4.73 ± 0.57	1	0.73	0.77	0.06	0	–	44	23.2	17 (9–36)
P3	3	11 June 2010	27	96.3	73	4.56 ± 0.47	0	0.75	0.78	0.03	0	–	50	14.2	28 (16–50)
P5	5	24 June 2010	27	92.1	89	4.89 ± 0.62	2	0.79	0.80	0.02	0	–	36	10.3	39 (24–73)
P8	8	15 July 2010	23	86.3	73	4.61 ± 0.61	3	0.69	0.77	0.10	0	–	38	15.0	27 (15–49)
P10	10	28 July 2010	17	87.4	68	4.79 ± 0.54	2	0.77	0.80	0.03	0	–	16	11.8	34 (15–202)
A	–	2010	35	100.0	101	4.93 ± 0.66	2	0.76	0.78	0.03	0	–	45	7.6	53 (34–87)
SGSL	–	2001–2010	235	99.6	155	4.81 ± 0.55	23	0.76	0.78	0.02	–	–	–	–	–

n total number of individuals sampled, *% LC* percentage of loci coverage, $_{T}n_{A}$ total number of alleles, $_{MWL}AR \pm SE$ mean within-locus allelic richness ± SE based on four individuals, $_{T}n_{PA}$ total number of private alleles, H_O Nei's (1978) observed heterozygosity across loci, $_{NB}H_E$ Nei's (1978) non-biased expected heterozygosity across loci, F_{IS} inbreeding coefficient across loci, n_{FS} number of potential full-siblings (Wang and Santure 2009; Wang 2011), $_{M}P_{FS}$ mean probability of correct assignments, n_{HS} number of potential half-siblings (Wang and Santure 2009; Wang 2011), *% sibship* percentage of pairs sharing at least one probable parent, N_{bEST} estimated number of breeders (Wang 2009), *CI* confidence interval

[a] *Letters* indicate stage of development [early veliger larvae < 160-μm shell length (*E*), late veliger larvae > 160-μm shell length (*L*), post-larval recruits (*P*)]; *numbers* indicate week of sampling

Table 3 Relative performance [bias and residual mean-squared error (*RMSE*)] of seven different relatedness estimator for six variable levels of kinship and true relatedness (R_{xy}): mixed dyads; unrelated dyads; second-cousin dyads; first-cousin dyads; half-siblings and double-first-cousin dyads; and full-siblings

Relatedness estimator	Mixed dyads ($R_{xy} = 0.257$)		Unrelated ($R_{xy} = 0.000$)		Second cousins ($R_{xy} = 0.031$)		First cousins ($R_{xy} = 0.125$)		Half-siblings double cousins ($R_{xy} = 0.250$)		Full-siblings ($R_{xy} = 0.500$)	
	Bias	RMSE	Bias	RMSE	Bias	RMSE	Bias	RMSE	Bias	RMSE	Bias	RMSE
TrioML (Wang 2007)	0.015[a]	0.148[a]	0.047	0.094	0.065	0.143	0.019[a]	0.144[a]	−0.022[a]	0.167[a]	<0.001[a]	0.158[a]
Wang (Wang 2002)	0.234	0.280	0.247	0.283	0.276	0.319	0.246	0.294	0.213	0.260	0.209	0.255
LynchLi (Lynch 1988; Li et al. 1993)	0.304	0.338	0.316	0.350	0.362	0.389	0.313	0.349	0.285	0.319	0.262	0.295
LynchRd (Lynch and Ritland 1999)	0.125	0.217	0.152	0.188	0.159	0.202	0.135	0.225	0.081	0.210	0.145	0.261
Ritland (Ritland 1996)	−0.140	0.221	0.018[a]	0.026[a]	−0.006[a]	0.026[a]	−0.078	0.140	−0.190	0.224	−0.395	0.415
QuellerGt (Queller and Goodnight 1989)	0.272	0.312	0.285	0.317	0.325	0.355	0.284	0.331	0.251	0.292	0.239	0.279
DyadML (Milligan 2003)	0.064	0.175	0.066	0.131	0.093	0.177	0.060	0.174	0.042	0.190	0.080	0.182

[a] The best estimator at each variable for each level of kinship

than randomly expected whenever 95 % of values of the $\Delta_{MIP}R_{xy}$ distribution were lower than the observed value of $\Delta_{MIP}R_{xy}$. *P*-values were determined using an inverse cumulative distribution function and the nominal value of 5 % was adjusted to 0.09 % following a sequential Holm–Bonferroni procedure (Holm 1979). For within-sample relatedness analyses, the term kin aggregation was used similarly as in Veliz et al. (2006) and defined an aggregation of larvae that are significantly more related to each other irrespective of their level of kinship.

A maximum-likelihood sibship assignment procedure using multi-locus genotype data (Wang and Santure 2009) was carried out as implemented in COLONY version 2.0.2.2 (Jones and Wang 2010). This analysis infers sibship by assuming that all individuals present within each sample are offspring issued from unknown sets of unrelated candidate mothers and candidate fathers. Offspring pairs can either be full-sibs (sharing both parents), half sibs (sharing only one of two parents) or unrelated to each other (sharing no parents). The method also works under the assumptions that markers are at linkage and Hardy–Weinberg equilibrium. Assignments were assisted by accounting for the allelic frequencies of each marker as previously detected within the SGSL genetic cluster (St-Onge et al. 2013).

The number of breeders (N_b) statistic gives an estimate of the actual number of adults responsible for producing all larvae in a given sample. Because samples comprised individuals belonging to the same age group and issued from a single breeding cycle and/or spawning season, N_b could be calculated by estimating the effective population size (N_e) statistic obtained from each sample (Hare et al. 2011). This was achieved using a maximum-likelihood sibship assignment method assuming random mating (Wang 2009; Wang and Santure 2009) as implemented in the software COLONY version 2.0.2.2 (Jones and Wang 2010). For comparison purposes, estimates of N_b were also obtained with the NeEstimator version 2.0. software (Do et al. 2014) using other methods such as the linkage disequilibrium method (Waples and Do 2008), the heterozygote excess method (Zhdanova and Pudovkin 2008) and the molecular co-ancestry method (Nomura 2008).

Results

Sample characteristics

Descriptive genetic characteristics summarized across all seven microsatellite loci are presented in Table 2. The genetic characteristics of each combination of loci and sample pertaining to analyses performed with MICRO-CHECKER (Van Oosterhout et al. 2004), genetic variability (heterozygosity, private alleles) and Hardy–Weinberg and linkage equilibrium are summarized in Online Resource 8.

Temporal genetic structure

Although the global multi-locus Δ_{ST} statistic (Weir and Cockerham 1984) indicated significant genetic structure (global $\Delta_{ST} = 0.003$; $P = 0.001$), the pairwise Δ_{ST} procedure did not reveal evidence of significantly different allelic frequencies among any of the 66 possible sample pairings based on the Bonferroni-adjusted nominal level of 0.08 % (Online Resource 9). Only two sample pairings (L3–P5 and P5–SGSL) showed *P*-values below 5 %. Global multi-locus Δ_{ST} values for early veliger larvae (*E*), late veliger larvae (*L*) and post-larval recruit (*P*) samples

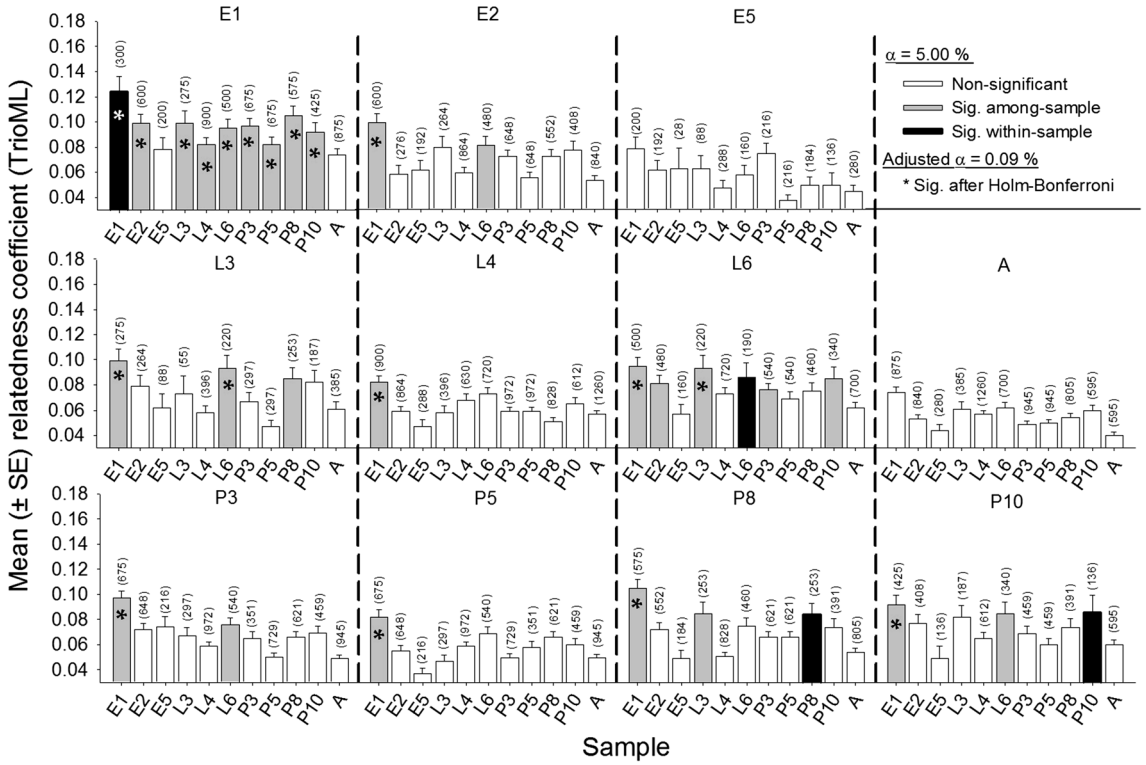

Fig. 1 Average (± SE) within-sample and among-sample individual pairwise relatedness coefficients ($_{IP}R_{xy}$) using the TrioML estimator (Wang 2007) as implemented in the COANCESTRY version 1.0.1.2. software (Wang 2011). Sample sizes representing the number of dyads analyzed are presented *in parentheses above each bar*. Pairings of larval and post-larval samples of softshell clams (*Mya arenaria*) that significantly deviated from the null hypothesis ($\alpha = 5$ %) of no difference in average pairwise relatedness (based on 1,000 bootstrap resamplings) are represented with *black* (within-sample comparisons) and *gray bars* (among-sample comparisons). See Online Resource 10 for additional information regarding specific *P*-values. *Letters* indicate stage of development [early veliger larvae <160-μm shell length (*E*), late veliger larvae > 160-μm shell length (*L*), post-larval recruits (*P*)]; *numbers* indicate week of sampling

grouped individually were also non-significant, amounting to −0.011 ($P = 0.231$), 0.002 ($P = 0.194$) and 0.004 ($P = 0.154$), respectively. Mean pairwise D_{EST} values (Jost 2008) across all loci and samples amounted to 0.025. In total, six sample pairings showed higher pairwise D_{EST} values than the upper limit of the 95 % confidence interval of 0.084, with three for each E5, L3 and P5 samples (where numbers indicate week of sampling) (Online Resource 9).

Genetic diversity

Gene diversity statistics ($_{MWL}$AR, H_O and $_{NB}H_E$) showed little evidence of reduced genetic variation in larval samples, as values were similar to both A and SGSL samples (Table 2). Mean within-locus allelic richness ($_{MWL}$AR) based on four diploid individuals did not vary significantly among samples ($F_{11,72} = 0.141$; $P = 0.999$). None of the Tukey pairwise comparisons of $_{MWL}$AR involving both A and SGSL was significant (mean *P*-value of 0.999; $n = 21$). Values for H_O and $_{NB}H_E$ for both A and SGSL samples were generally within the range observed for all larval and post-larval recruit samples (Table 2).

Within- and among-sample relatedness

Within-sample means of $_{IP}R_{xy}$ coefficients were significantly higher than those of randomly grouped individuals in the E1, L6, P8 and P10 samples (Fig. 1). Among-group means of $_{IP}R_{xy}$ coefficients showed that 23.6 % of samples were, on average, more related to each other than randomly expected (Fig. 1). Eight of these implicated the very first veliger larvae (E1) to be produced in the spawning season, which was shown to be more related than randomly expected with more than 80 % of all samples. Seven out of 16 sample pairings (E2–L6, L3–P8, L6–L6, L6–P3, L6–P10, P8–P8 and P10–P10) showing significant $_{IP}R_{xy}$ coefficients had *P*-values above the Holm–Bonferroni-adjusted nominal level of 0.09 % (Holm 1979). However, all sample pairings involving the E1 sample had *P*-values well below the adjusted nominal level (Online Resource 10).

Sibship assignment

A total of six full-sib (n_{FS}) and 406 half-sib (n_{HS}) dyads were estimated to be present across samples (not including SGSL) (Table 2). All six full-sib pairs were exclusively estimated to belong to E samples: five in the first early veliger (E1) sample with a mean probability of 52 % and one in the second early veliger (E2) sample with a probability of 100 %. The mean percentage of individual pairings within a sample that share at least one parent (% of sibship) was averaged across all ten larval and post-larval recruit cohorts and amounted to 16.1 %, while it amounted to only 7.6 % in the A sample (Table 2).

As 64 % of the mean pairwise relatedness coefficients ($_{IP}R_{xy}$) calculated within and among samples were situated between 0.06 and 0.10 (Fig. 1), levels of kinship were considered to be somewhere between first ($R_{xy} = 0.125$) and second cousins ($R_{xy} = 0.031$) (Falconer and Mackay 1996). These levels of kinship have withdrawn overestimations of relatedness between 1.9 and 6.5 % using the TrioML estimator (Wang 2007) (Table 3). Furthermore, biases of TrioML were observed to be nearly absent for half-sibs ($R_{xy} = 0.250$) and full-sibs ($R_{xy} = 0.500$), two levels of kinship observed in this data set (Table 2). The lowest sample sizes were E5 ($n = 8$), L3 ($n = 11$) and P10 ($n = 17$), creating a possibility of 28, 55 and 136 dyads, respectively, inside of which six (21.4 %), 13 (23.6 %) and 16 (11.8 %) half-sibling relations were estimated (see n_{HS} and % of sibship in Table 2). While these data suggest that small sample sizes generated higher percentages of sibship, a simple regression analysis carried out with only within-sample statistics showed that the relationship between the number of observed half-sibling relations and the number of possible dyads is a linear one ($r = 0.854$; $P = 0.008$). With such a correlation, sample size is thus not likely to affect the overall level of average relatedness within or among samples. Therefore, evaluations of relatedness were considered relatively accurate and relevant.

Temporal variance in reproductive success

As the reproductive cycle progressed, the mean number of breeders (N_b) responsible for producing all individuals in samples ranged between 17 (L3 and L6) and 39 (P5) (Table 2). In addition, N_b fluctuated similarly for all development stages (i.e., E, L and P) with an approximate 1.7-fold increase from the first to the second time point and a subsequent decrease back to near-original values from the second to the third time point. The time period separating samples E1–L3, E2–L4 and E4–L5 is consistent with a mean pelagic larval duration of 10–35 days for *M. arenaria* (Shanks 2009). These corresponding samples showed similar mean values of N_b (Table 2). Estimates of N_b obtained using either the linkage disequilibrium (Waples and Do 2008), the heterozygote excess (Zhdanova and Pudovkin 2008) or the molecular coancestry (Nomura 2008) methods implemented in NeEstimator version 2.0. (Do et al. 2014) were all considerably higher than those obtained with the sibship assignment method (Wang 2009) (Online Resource 11).

Discussion

The main goals of this study were: (1) to provide a detailed picture of the temporal variation of genetic diversity through larval development of the softshell clam *M. arenaria*, (2) to assess whether we could observe the presence of genetic patchiness within the first stages of its life cycle, and (3) to use molecular tools to track larval pools and evaluate if the mechanisms modulating recruitment could be defined more clearly. Microsatellite markers represent ideal molecular tools to achieve these goals. They can infer family relations between individuals because they provide information at single independent loci, are co-dominant, are usually hypervariable, are characterized by short DNA sequences and are easily amplifiable (Selkoe and Toonen 2006; Pemberton 2008; Jones et al. 2010). It is, however, important to carry out parentage analyses, relatedness statistics and sibship assignment procedures using a large number of highly polymorphic markers to prevent the overestimation of family relations between individuals (Pemberton 2008; Jones et al. 2010). Given the relatively low number of markers used in this study, the least biased and the most relevant statistical procedures available were employed as assessed from previously published data-simulation reports (Wang 2007, 2009; Wang and Santure 2009). Wang's (2009) sibship assignment estimator was used here to obtain N_b estimates because it was shown to be more accurate than other methods for data sets with smaller sample sizes. It also provided confidence intervals without infinite values which seemed more realistic given the ecological setting being studied. Rather than focusing on absolute values, N_b estimates are only used here to compare relative differences between samples and to determine if temporal variance in reproductive success can be observed within a single reproductive season.

Temporal genetic structure

For the first time, we demonstrated in a bivalve species that all larval pools issued from partial spawning were genetically homogeneous among themselves, throughout larval development and compared with the breeding population. A study involving three to four temporal samples of larval Pacific oysters (*C. gigas*) collected at two different

sampling sites over a single spawning pulse (7–11 days) showed two samples with significant genetic heterogeneity (Li and Hedgecock 1998). However, these individuals were not genotyped with highly polymorphic microsatellite markers but rather with single-strand conformational polymorphisms with one common haplotype dominating 53–85 % of all samples. Furthermore, spawning profiles of *C. gigas* recorded during that study were more representative of a MSS event, while this study confirmed the presence of the PSS strategy for *M. arenaria* in the studied environment with the observation of many spawning events and sequential pools of larvae (Table 1; Online Resources 2, 5). Another important difference between this study and Li and Hedgecock's (1998) is that they only observed larval *C. gigas* samples comprising individuals in their first larval developmental stages and not from later stages, such as new settlers and late-surviving recruits.

Sample relatedness

While one would normally conclude from these abovementioned temporal genetic structure results that SRS did not govern the observed pattern of recruitment, assessments of mean pairwise relatedness both within and among samples have clearly suggested otherwise. In fact, evidence for variance in reproductive success was found in the form of significantly related samples of larvae and post-larval recruits in an overwhelming 40 % of all samples (Fig. 1). Such observations are suggestive of kin aggregation, high relatedness and sibship in larval and post-larval recruit pools and should be considered extreme cases of SRS (Hedgecock and Pudovkin 2011), which until now had only been reported for a small number of marine organisms, such as the hermaphroditic northern acorn barnacle *Semibalanus balanoides* (Veliz et al. 2006), the California spiny lobster *Panulirus interruptus* (Iacchei et al. 2013), the cnidarian *Pelagia noctiluca* (Aglieri et al. 2014) and some species of small reef fishes (Jones et al. 2005; Selkoe et al. 2006; Christie et al. 2010; Bernardi et al. 2012; Horne et al. 2013).

A total of six full-sib dyads were estimated to be present within the first two E samples. To our knowledge, these results are unprecedented and represent the first observations of kin-aggregation patterns within larval samples of a natural population of marine bivalve. It is, however, important to underline and understand the limitations surrounding sibship-reconstruction methods. Based on previously published simulation procedures with known levels of kinship [see top left graph of Fig. 1 and left graph of Fig. 2 in Wang and Santure (2009)], and assuming equal allelic frequencies for all polymorphic alleles at seven microsatellite markers, the maximum-likelihood method that was used in this study for reconstructing sibship structure within samples is predicted to be approximately 65 % accurate. However, this method seems to be the more accurate, more powerful and more conservative than other commonly used methods such as simple pairwise relatedness procedures, which can often be considered unreliable due to the fact that they do not include all individuals in their total reconstruction algorithm with each individual pair being processed independently (Wang and Santure 2009). Bernardi et al. (2012) used pairwise relatedness methods to assess the presence of sibship in *Dascyllus trimaculatus* recruits, using an R index (Queller and Goodnight 1989) threshold of 0.5, above which individuals in a pair were considered to be either full- or half-sibs. Based on a similar modified procedure with the TrioML relatedness estimator (Wang 2007) and discarding all individuals that were missing genotypes for at least three loci, several instances of pairwise relatedness indices above 0.5 were also found within samples in this study (e.g., 13 within E1, six within P8 or two within P10). Wang and Santure's (2009) sibship reconstruction algorithm was, however, chosen here in order to remain as conservative as possible and limit the overestimation of sibship within the studied system.

It is also interesting to note that the probability of all full-sib dyads to be actual full-sibs was much higher than the probability of them being either half-sibs or unrelated. For example, one full-sib cluster detected in the E1 sample comprised three individuals (hereafter *X*, *Y* and *Z*). The probability that *X*, *Y* and *Z* were all full-sibs among each other was only 48.7 %. But the probabilities that only two of them were full-sibs (e.g., *X* and *Y*) with the third one (*Z*) being unrelated, or that all three individuals (*X*, *Y* and *Z*) were unrelated, were even lower, amounting to only 20.7 and 1.2 %, respectively. In spite of Wang and Santure's (2009) method being less than ideally accurate considering the limited number of genetic markers used in this study, such results are supportive of the general finding that relatedness and kin-aggregation patterns within larval cohorts of *M. arenaria* sampled in Bouctouche Bay in 2010 were considerably high.

Larvae observed to be kin-aggregated inside the first early veliger sample (E1) might have resulted from a small number of precocious breeders in the very beginning of the reproductive season. Spawning was shown to be dominated by only a few individuals in some marine species (Borrell et al. 2008; Lemay and Boulding 2009; Li and Li 2011). It is also possible that limited lifespan/transport distance of gametes (Lasker et al. 2008) coupled with a relatively smaller number of breeders reduced the number of parental partnerships and increased average relatedness within the E1 sample. However, the numbers of breeders (N_b) estimated with the effective population size (N_e) for E1 and all other veliger samples do not support this hypothesis. E1 was rather shown to have a fairly similar N_b to E5, L3 and

L6 (Table 2). Furthermore, no reduction in gene diversity and no differences in allelic frequencies were observed between E1 and adult reference samples, suggesting that these breeders were genetically representative of the larger population or that they represented a more genetically diverse group of breeders (Rios et al. 1996; Bierne et al. 1998). The fact that these closely related larvae stayed close to one another in the water column could be due to kin-recognition processes or to the presence of dispersal kernels (e.g., Siegel et al. 2003).

Another possible explanation for the presence of larval kin aggregation could be related to the increasing surface water temperature (Table 1) inducing temporal differences of reproductive behavior throughout the spawning season. Such differences have already been described for other species of marine bivalves (e.g., Myrand et al. 2000; Cyr et al. 2007; Enriquez-Diaz et al. 2009). For example, sea scallops *P. magellanicus* showed differential degrees of spawning synchrony depending on the year of sampling due to annual differences in environmental conditions (Langton et al. 1987). These observations suggested that, depending on environmental conditions, breeders seemed capable of ensuring reproductive success by switching back and forth between evolutionary stable spawning strategies, showing MSS behaviors under certain conditions and longer protracted spawning behaviors under different conditions (Langton et al. 1987; Dukeman et al. 2005). It is thus possible that a very small fraction of precocious breeders opted for the alternative reproductive strategy of releasing their entire gonadic contents in one single MSS event, explaining why so many full-sibs were detected in earlier samples but not in subsequent samples. If true, these individuals would represent a small fraction of the breeding population since the temporal assessment of GSI clearly indicated the presence of partial spawning over a period of 6 weeks (−1 to 5 weeks post-FSE). However, the E1 sample was shown to be genetically related to more than 80 % of all other larval samples, suggesting that the breeders responsible for producing E1 larvae likely continued to reproduce by partial spawning throughout the entire reproductive season.

Larval retention

The classic definitions of "local retention" (locally produced settlement relative to total amount of larvae released) and "self-recruitment" (locally produced settlement relative to the total settlement from elsewhere) (Botsford et al. 2009; Christie et al. 2010; Berumen et al. 2012) both involve knowing whether recruits are directly linked with breeders from their settlement location. Christie et al. (2010) were able to show larval retention and self-recruitment by genotyping a sample of adults and newly settled recruits of the bicolor damselfish (*Stegastes partitus*) from which caudal fin tissue was collected in a non-lethal fashion. Logical constraints prevented the use of similar methods with *M. arenaria* individuals. Considering the endobenthic lifestyle of this species, collecting tissue for DNA extraction without being invasive and without removing *M. arenaria* from its burrow would have been virtually impossible.

Given that linking recruits with breeders through parentage analyses was impossible, it is argued that results presented here have come really close to demonstrating genuine larval retention and self-recruitment. Larvae collected in the E1 sample were D-shaped, representing the first veliger stage following the trochophore stage (24 h post-fertilization) and were thus only a few days old when collected. These newly spawned larvae were shown here to be retained at the same location for more than 95 % of their entire larval development period, i.e., from their first veliger stage (D-shaped) to the very end of their post-larval period (approximately 1 mm of shell length) (Fig. 1). Given that only one more population of softshell clams was found at the opposite side of the bay, one can safely assume that these larvae were also retained close to their spawning grounds during the first 5 % of their life cycle.

The absence of larval dispersal studies in the Bouctouche Bay and surroundings makes it impossible to state that larval dispersal at this site is achieved similarly or differently from that of other systems. Bouctouche Bay is characterized by a 1.5-km-wide opening on the Gulf of St Lawrence (Online Resource 1), outside of which surface waters are able to travel at speeds between 40 and 50 cm/s, so one cannot eliminate the possibility of larval transport inside or outside of the bay. However, larval and post-larval samples were collected in the most secluded part of the bay, i.e., at the very base of a 12-km-long sand dune. This prominent topographical feature most likely acted as an important barrier to larval dispersal and provides a good explanation for the observation of larval retention.

Furthermore, some related sample combinations such as E1–E2, E1–L3, E1–L4 revealed the ability of molecular tools to track larval pools throughout ontogeny in a larval retention setting. High among-sample relatedness observed among sample combinations such as E1–L6, E2–L6 or L3–L6, however, could not be explained by ontogeny since *M. arenaria* larval development usually lasts only between 1 and 4 weeks (Shanks 2009). These results are rather explained by the presence of partial spawning, which was confirmed with the GSI index (Online Resource 2).

One of the main challenges still facing marine ecologists today is the quantification of larval dispersal between discrete benthic populations relative to larval retention. These assessments are critical to improve recruitment models, increase our knowledge of marine population ecology and allow optimal selection of marine protected areas

(Berglund et al. 2012). Several studies showed compelling evidence of larval retention with a wide variety of multidisciplinary and indirect methods such as physical modeling of surface currents linked with temporal variance in larval distributions (e.g., Lamare 1998; Morgan et al. 2009) and elemental fingerprinting (e.g., Becker et al. 2007). But as shown here, relatedness statistics linking pools of recruits sampled at different times represent a more direct method for assessing the level of retention in a given marine system (Hedgecock 2010) and are likely to be of great use to conservation biologists wishing to properly establish marine protected areas (Palumbi 2004).

Recruitment success

It is also clear that the very first partial spawn that occurred at the beginning of the season was the most successful in contributing to recruitment. More than 80 % of all samples were significantly more related to the first pool of newly spawned larvae (E1) than randomly expected, which included all post-larval recruit samples collected throughout the season from P3 to P10. Only P8 and P10 showed significant levels of within-sample relatedness with values of approximately 0.085 compared with approximately 0.061 for P3 and P5. This suggests that few larvae produced during later spawns actually survived until the recruitment stage determined at 10 weeks post-FSE. This conclusion is also supported by the fact that P10 post-larval recruits were twice as large as all other post-larvae from P3, P5 and P8 and could not have been issued from recent spawns (Online Resource 5).

These results confirm the importance of the very first larval pool for shaping the local recruitment of this site in this spawning season. The optimal timeframe during which surviving recruits were produced was thus at the very beginning of the spawning season, suggesting that oceanographic conditions present during the first partial spawn likely played an important role in the recruitment success of resulting larvae. Such conditions can include an optimal surface water temperature (Table 1), favorable trophic conditions and the relative absence of predators or lower competition for various important resources. It is also possible that the first gametes released by breeders in a spawning cycle are of better quality than those released later in the course of the cycle. The overall quality of lipid profiles in female eggs released during the course of a single reproductive cycle could potentially be interesting to assess in future studies.

Importance for assessing relatedness

It seems somewhat paradoxical to have observed an absence of SRS due to temporal variations in allelic frequencies when such strong temporal relatedness patterns suggest the opposite (Hauser and Carvalho 2008). However, it can also only mean that connectivity can be limited on ecological timescales in places where gene flow was sufficient enough to have homogenized allelic frequencies over several thousands of generations (Hedgecock 2010). While the vast majority of marine invertebrate studies reporting an absence of SRS have based their conclusions on statistical analyses solely focused on the level of differentiation between samples, this study warrants the importance of looking at SRS from the opposite perspective: focusing on how much samples are actually related to each other (Hauser and Carvalho 2008; Hedgecock 2010; Iacchei et al. 2013). This approach should be useful in increasing detection limits of connectivity in broadcast-spawning marine invertebrate populations with homogeneous allelic frequencies and give more insights on the factors responsible for recruitment.

In a recent literature review, Hedgecock and Pudovkin (2011) enumerated several predictions of SRS consequences on the genetic population structure of highly fecund marine species:

1. The ratio of effective population size (N_e) to the actual number of individuals in the population (N) should be extremely small ($N_e/N \ll 0.01$).
2. The number of breeders and individual reproductive success should be variable within and among spawning periods.
3. Random genetic drift should occur simultaneously with single recruitment cycles thus causing significant shifts in allelic frequencies among cohorts of recruits sampled within a given population.
4. Genetic diversity of recruits such as allelic richness should be significantly lower than in the adult population of origin.
5. In extreme cases, individuals within cohorts should be significantly more related to each other than in groups of randomly selected individuals originating from the same population.

Only the last four predictions were tested in this study as the actual number of individuals in the studied population (N) was not available and would have been very difficult to obtain. But highly fecund marine organisms with type III survivorship curves such as *M. arenaria* (Brousseau 1978) typically have very low N_e:N ratios. Out of the four tested predictions in this study, the third and fourth predictions did not seem to be reflected by the results. However, the fact that the percentages of sibship observed in larval and post-larval recruit samples were higher than those observed in adults (Table 2) still provides good evidence for the fourth prediction. Also, if only a few

isolated cohorts would have shown high within-sample sibship/relatedness, it would have been possible to observe genetic heterogeneity among samples through reduced genetic diversity. This was not the case here since both high within-sample and high among-sample relatedness was observed across the whole sampling period. This observation was most likely due to PSS breeders being active throughout the entire reproductive period, expectantly causing the genetic diversity of sampled cohorts to be homogeneous. More studies using molecular tools coupled with a monitoring of partially spawned larval pools throughout larval development are warranted to assess whether the patterns reported here are consistent among years, sampling sites and species. The overall lack of information in the ecological literature regarding MSS and PSS strategies also calls for more empirical studies on the factors regulating their mechanisms.

Author contribution statement PS conceived the experiments. PS, RT and JMS designed the experiments. PS performed the experiments and analyzed the data. PS, RT and JMS wrote the manuscript.

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