

ARTICLE

Coastal and Marine Ecology

Evolutionary and ecological insights from vital staining of bivalve oocytes: A Red Queen at the sweepstakes?

Peter G. Beninger¹  | Daphné Chérel¹ | Gaël Le Pennec²  | Sandra E. Shumway³ 

¹Laboratoire de Biologie Marine, Faculté des Sciences, Université de Nantes, Nantes Cedex, France

²Laboratoire de Biotechnologie et Chimie Marines, Université de Bretagne Sud, Lorient, France

³Department of Marine Sciences, University of Connecticut, Groton, Connecticut, USA

Correspondence

Peter G. Beninger

Email: peter.beninger@univ-nantes.fr

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Abstract

To determine the proportion of inviable oocytes that are spawned along with normal oocytes in marine bivalves, we turned to a time-honored technique not previously used on invertebrate oocytes: the neutral red vital stain. Despite the difficulty in obtaining induced spawns from field-collected *Cerastoderma edule* (L), the results and insights from this simple procedure to answer a simple question were unexpected and far-reaching. Mean proportions of inviable oocytes in the spawns of cockle *C. edule* ranged from 35% to 85%, with the vast majority represented by atresic (autolyzing) oocytes. These levels are in agreement with previous quantitative histological observations of prespawning atresia in both *C. edule* and the Manila clam *Tapes philippinarum*. Such elevated prefertilization inviability mirrors the known high level of postfertilization inviability in bivalves. Together, these high levels of inviability may account for much of the early mortality in the type III survivorship curves typical of marine bivalves. The combination of high fecundity and high oocyte inviability suggests a Red Queen-type reproductive dilemma in these species. In addition, the great degree of interindividual variation observed in oocyte inviability suggests that cockles, and perhaps other intertidal bivalves, present a Sweepstakes Reproductive Strategy, in which highly variable individual fecundity confronts a highly variable environment. The neutral red technique promises to be a useful tool for investigating prespawning inviability in other marine taxa, as well as for aquaculture, conservation, restocking, ecophysiology, and environmental monitoring.

KEYWORDS

atresia, bivalves, neutral red, oocytes, Red Queen dilemma, Sweepstakes Reproductive Strategy

INTRODUCTION

All of the commercially important marine bivalves (oysters, cockles, mussels, scallops, and clams) typically

spawn hundreds of thousands or millions of microscopic oocytes (Cáceres-Puig et al., 2016; Galinou-Mitsoudi & Sinis, 1994; Park & Choi, 2004) and are characterized by very high early life stage mortalities (Plough, 2018;

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Plough et al., 2016). Both fecundity and early life stage mortality are crucial determinants of recruitment to a natural population, to a fishery, or to a shellfish hatchery. The spawner–recruit function is central to stock–recruitment (SR) analysis, and accurate estimates of spawner fecundity are critical components of this function (Maunder & Thorson, 2019). In contrast to low-fecundity species, in which a relatively deterministic SR model can be developed, such models cannot be used for high-fecundity species such as bivalves (Koslow, 1992), necessitating the measurement of fecundity when this datum is required.

Fecundity is usually defined as the number of viable oocytes spawned by a female of a certain age or length–weight class (more specifically F_{real} , realized fecundity, as opposed to F_{pot} , potential fecundity). While this may be an accurate theoretical definition, it is difficult to put into practice, especially for species such as bivalves, whose oocytes are microscopic, and usually planktonic. To our knowledge, although F_{real} is routinely estimated for fish (e.g., Jennings et al., 2001), it has not yet been determined for any marine invertebrate. Obviously, an improved understanding of early life stage mortality, including oocytes, is a prerequisite to better estimations of recruitment and ultimately production.

Approximations of oocyte numbers at a given time may be obtained through induced spawning of bivalves (which is often difficult to achieve, and may only be partial, outside of hatchery conditions), or gonad stripping (which is often incomplete and nonrepresentative of the natural process). Moreover, oocyte numbers do not translate directly to fecundity, unless some measure of oocyte viability is known. The most widespread indicator of cellular viability is the neutral red (NR) vital stain, first developed by Ehrlich (1894), and used extensively since the 1970s, both in the medical sciences (e.g., Liu et al., 2018) and for marine plankton (Da Luz et al., 2016; Dressel et al., 1972, and see Beninger et al., 2021 for review).

Previous work has highlighted the ubiquitous, often-overlooked presence of atresic (degenerating) oocytes of all gametogenic stages within the gonads of marine bivalves (see Beninger, 2017; Chérel & Beninger, 2017, 2019; Chérel et al., 2020 for reviews and references). Despite the prolific scientific literature on reproductive cycles in high-fecundity marine organisms, only one study to date has examined the viability of their spawned oocytes (Beninger et al., 2021), that is, what proportion are actually capable of being fertilized and beginning development?

We recently used the NR vital staining technique on the spawned or stripped oocytes of four commercially important marine bivalve species: Pacific oyster *Crassostrea gigas* Thunberg, mussel *Mytilus edulis* L, clam *Tapes*

philippinarum (Adams and Reeve, 1850), and cockle *Cerastoderma edule* (L) (Beninger et al., 2021 and present study). Although the mechanism of NR vital staining has been the object of a great deal of unverified conventional wisdom, it is known to rapidly accumulate in intact lysosomes, and to subsequently re-diffuse into the cytosol much more slowly (Patetsini et al., 2013).

MATERIALS AND METHODS

In contrast to the simplicity of the NR technique, obtaining spawns of female oocytes from field-harvested adults proved to be extremely arduous: 300 *C. edule* were sampled and used for spawning during the peak spawning months between 2019 and 2021; as there is no macroscopic sexual dimorphism either in the shell or in the soft tissue, it may be assumed that approximately half of these dioecious individuals were female (Maia et al., 2021; Twomey & Mulcahy, 1988). The spawning induction technique was chosen to approximate exaggerated natural conditions, particularly temperature and immersion–emersion periods (Honkoop & Van der Meer, 1998): During spawning (immersion) trials, thermal shock cycles of 10°C amplitude and 2-h duration were implemented (from 12 to 22°C) up to five times over a maximum 10-h period. Emersion was simulated by loosely wrapping the animals in seawater-impregnated toweling at 7°C for 8 h (slightly below spring nighttime temperatures of 12–14°C). The procedure required continuous attentive observation, often over the entire 10-h immersion period, as spawning occurred in very short and small bursts of several dozen microscopic, near-transparent oocytes at a time, and oocytes not recovered, stained, and photographed within 30 min were often unusable. Over all of the assays between 2019 and 2021, 20 of these females spawned, for a success rate of 13%.

After exposure to the NR stain for 2–3 min at 5 mg ml⁻¹, normal, live oocytes were successfully stained (and went on to become NR-stained larvae), dead oocytes were unstained, and an intermediate category of “not yet dead” oocytes was stained to varying degrees, but could easily be distinguished by their abnormal cellular characteristics (Figure 1). These latter oocytes comprised both atresic and malformed cells, which were de facto inviable.

RESULTS AND DISCUSSION

Despite the challenge of spawning induction in field-collected animals, these first quantifications of spawned oocyte categories yielded several interesting and

surprising results. It was shown that a simple staining procedure allows the ready differentiation of normal and inviable bivalve oocytes. The proportion of inviable oocytes in spawns was considerable; for the cockle *C. edule* on three sampling dates in 2019 and 2021, the mean percentage of inviable oocytes was 70, 85, and 35 (Table 1). Despite the difficulty of spawning induction in field-collected animals, and hence the relatively low number of spawns reported here, the effect size is quite large (35%–85% inviable oocytes); for a discussion of effect size and replication in mudflat ecology, see

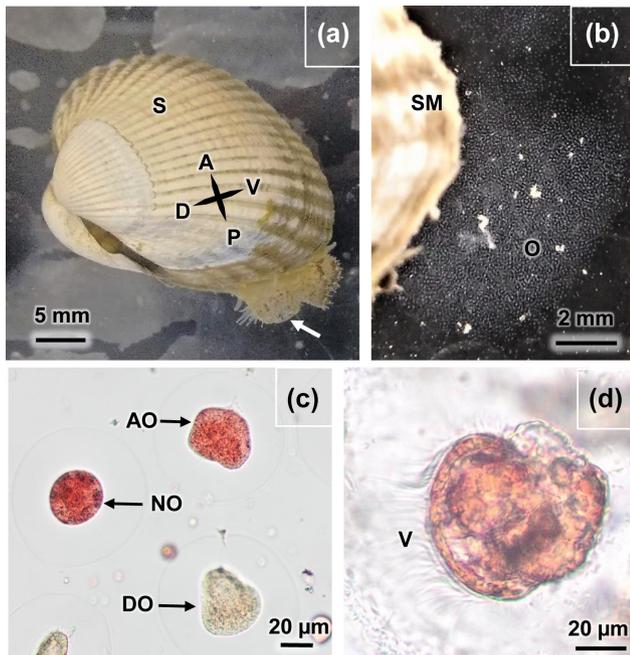


FIGURE 1 Spawning and early development of neutral red-stained cockle *Cerastoderma edule* oocytes. (a) Female cockle resting on right valve, showing exhalant region from which oocytes are spawned. Anatomical extremities: A, anterior; D, dorsal; P, posterior; S, shell left valve; V, ventral; (b) detail of exhalant region, siphons retracted, beyond shell margin (SM) showing spawned oocytes (O); (c) neutral red-stained spawn, showing normal (NO), atresic (AO), and dead (DO) oocytes; (d) transitional trochophore-veliger larva developing from neutral red (NR)-stained oocyte, 24 h postspawning; V, cilia of developing velum

TABLE 1 Mean percentage and range (in parentheses) of normal, atresic/abnormal, dead, and total inviable oocytes in spawns of the cockle *Cerastoderma edule*, spring 2019 and 2021

Sampling date	N	Normal	Atresic or abnormal	Dead	Total inviable
17 April 2019	3	30.0 (28.3)	65.6 (32.2)	4.3 (3.9)	70.0 (28.3)
2 May 2019	4	14.6 (19.4)	81.8 (23.0)	3.6 (7.4)	85.4 (19.4)
19 May 2021	13	65.2 (31.3)	21.3 (19.2)	13.5 (916.5)	34.8 (31.3)

Note: N, number of females for which spawns were obtained. Range is used as the measure of dispersion about the mean, due to the low number of spawning individuals in 2019 (Beninger et al., 2012). Total inviable = Atresic or abnormal + dead oocytes. Data from Beninger et al. (2021) and present work.

Beninger and Boldina (2018). Furthermore, the convergent data from previous prespawning quantitative histological observations (Table 2) support the inviable oocyte results.

Prior to these histological and live spawning results, such inviable oocyte numbers were unexpected, suggesting that high fecundity in these species may be accompanied by high genotypic oocyte inviability. A specific evolutionary process in which adaptations of one taxonomic group induce compensatory adaptations in an interacting taxonomic group has been called the Red Queen hypothesis (RQH; see Strotz et al., 2018; Van Valen, 1973). The RQH used Lewis Carroll's Red Queen scene from "Through the looking glass, and what Alice found there," in which the protagonists had to run as fast as they could in order to simply stay in the same place. Although its original transposition to biology has been typically restricted to interactions between taxonomic groups and has been both supported and strongly contested (e.g., Kerfoot & Weider, 2004; Vermeij & Roopnarine, 2013), the original metaphor can be usefully extended to situations where one adaptation, regardless of etiology, is largely negated by another

TABLE 2 Aggregated data for minimum atresic impact (defined as the percentage of mature oocyte volume fraction for which an atresic fate is known from histological profile) in two intertidal bivalve species, *Cerastoderma edule* and *Tapes philippinarum*, based on quantitative histological observations of the gonad, from two sites and years 2010–2018

Species	Minimum atresic impact	
	Total active gametogenesis	April–May
<i>Cerastoderma edule</i>	51.3% ± 10.8	58.4% ± 10.7
N	151	77
<i>Tapes philippinarum</i>	46.8% ± 11.4	43.8% ± 21.0
N	230	52

Note: Total active gametogenesis = individuals for which mature oocytes ≥20% of gamete volume fraction; April–May = aggregated data from all years for these 2 months, during which all atresia is prespawning. Data from Chérel and Beninger (2017) and Chérel et al. (2020).

character trait in the same species. Such a concept would thus also summarize cases where adaptations of a character are at least partially stymied by endogenous antagonistic processes, such that organisms may expend considerable energy simply to remain in the same adaptive “place.” To distinguish it from RQH, while acknowledging the common metaphor, we term this broader, overall concept the Red Queen Dilemma (RQD). In the present situation, the character of high fecundity appears to be accompanied by an antagonistic trait of high oocyte inviability within the bivalve species examined, evoking an RQD.

Prior to the NR stainings of spawned oocytes, it was not known whether atresic oocytes observed in gonad histological sections (Table 2) were all absorbed and recycled in the gonad, or whether some or all of them were released in the spawns. It now appears that most of the inviable oocytes present in the gonad at the time of spawning are released along with the normal oocytes. Bivalve fecundity estimates based on unstained, spawned, or stripped oocytes may therefore greatly overestimate F_{real} . Furthermore, the release of these inviable oocytes represents a potentially great energetic loss not previously recognized or considered in production models (e.g., Sarà et al., 2013).

While the high oocyte inviability levels brought to light in our work on oocyte atresia in bivalves may surprise most marine biologists, it should be noted that even greater levels of oocyte inviability throughout oogenesis are typical of higher vertebrates. In a review of this topic, it has been remarked that “...the number of germ cells enclosed in primordial follicles at birth is less than 20% (human, Baker, 1963) or less than 5% (cow, Erickson, 1966) of its peak number. This is solid evidence demonstrating that the normal fate for a female germ cell during oogenesis is death.” (Reynaud & Driancourt, 2000). The fundamental difference, however, is of course the internal fertilization and subsequent gestation of higher vertebrates, which vastly increases the probability of oocyte fertilization and successful early development. Given the already haphazard nature of fertilization in aquatic broadcast spawners, and the high level of subsequent mortality of the young stages (typical of these type III survivorship species), the level of oocyte inviability brought to light by the present work is indeed surprising.

In addition to the elevated proportion of inviable oocytes (i.e., prefertilization inviability) observed in this work, interindividual variability with respect to this characteristic was itself considerable (Table 1), suggesting that reproductive success among individuals is likely to be highly variable at the very outset, due to endogenous factors. A similar scenario has previously been described and explored in detail for *postfertilization* life stages in

bivalves (Plough, 2018; Plough et al., 2016), where the high mortality of early stages was shown to be due to genetic inviability (e.g., abnormal/malfunctioning larvae). The term Sweepstake Reproductive Success (SRS) was coined to characterize genotypic-driven, high early *postfertilization* life stage mortality, combined with high interindividual broodstock variability in this character (Hedgecock & Pudovkin, 2011). Although the SRS term was coined in the context of *postfertilization* observations, there is no reason why it cannot also be applied to similar processes acting upon oocytes prior to fertilization. The results of our study suggest that a SRS scenario exists at the *prefertilization* level, such that the entire oogenesis–spawning–ontogenesis (i.e., prefertilization–fertilization–postfertilization) sequence is played out as a “reproductive sweepstakes.” In such a framework, the high variability of the intertidal environment is matched or mismatched to the similarly variable F_{real} of the individual spawning female bivalves. The main consequence of this process is that most of these individuals have poor reproductive success, while relatively few have the capacity for disproportionately high reproductive success. Correlatively, the effective female population size is quite small, compared to the actual population size, and the resulting gene pool is likely to be reduced—which is exactly what is observed in most marine animal populations (Hedgecock & Pudovkin, 2011).

Numerous uses can be envisaged for the simple NR oocyte vital staining tool developed in this work. On a fundamental level, it is of considerable interest to elucidate the genetic basis for the Red Queen and SRS outcomes. On an applied level, the opportunities of combining induced spawning (despite its difficulty) rather than destructive gonad stripping, with selection of high F_{real} individuals, are important and numerous: (1) to better understand what drives the poor survivorship occurring in the shadowy zone between spawning and recruitment of most marine animals; (2) to identify high F_{real} broodstock in commercial species, and, provided the trait has a reasonable heritable component, select for commercial species brood lines with increasingly high F_{real} , especially those which have low hatchery success (Christie et al., 2014); (3) to use such genetically selected high F_{real} broodstock in restocking and conservation efforts; and (4) to better understand the effects of anthropogenic inputs, such as heavy metals and endocrine disruptors, and/or the effects of changes in water temperature, pH, or toxic algal blooms on bivalve oocyte viability, with a view to its eventual use in environmental monitoring and prevention. We encourage our colleagues working on other high F_{pot} marine taxa to similarly explore the possibilities of this technique.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Beninger et al., 2022) are available from Zenodo: <https://doi.org/10.5281/zenodo.6006705>.

ORCID

Peter G. Beninger  <https://orcid.org/0000-0002-1308-9986>

Gaël Le Pennec  <https://orcid.org/0000-0001-6640-2908>

Sandra E. Shumway  <https://orcid.org/0000-0003-1494-6297>

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