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Examining bivalve fecundity: oocyte viability revealed by Neutral Red vital staining

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Abstract

Estimation of realized fecundity (F_{real}, number of *viable* oocytes produced) is an essential, yet seldom-achieved element in the understanding of marine animal production and population dynamics. We used the Neutral Red (NR) vital stain to determine oocyte viability in spawns and gonad strippings of four species of commercially-important bivalves: *Cerastoderma edule* (L), *Crassostrea gigas* Thunberg, *Mytilus edulis* L, and *Tapes philippinarum* (Adams and Reeve). The utility of Trypan Blue as a complementary mortal stain was also assessed, and found to be unnecessary. Normal, live oocytes stained with NR, and were either spherical (mature oocytes) or pedunculated (immature oocytes); atresic/abnormal oocytes also stained with NR, allowing their ready recognition due to their abnormal shapes and cytoplasmic retraction. Dead oocytes did not stain with NR. Across the species studied, a considerable and highly variable proportion of spawned or stripped oocytes was either dead or non-viable; quantitative counts were performed for *Cerastoderma edule*, the only species for which > 5 spawns occurred. The high level and variability (34–85%) of dead or non-viable oocytes is consistent with a reproductive Red Queen dilemma, in which greater oocyte numbers do not translate to commensurately greater real fecundities, and also with a Sweepstakes Reproductive Success strategy, in which a large range of F_{real} confronts the considerable variability of intertidal environmental conditions. Neutral Red vital staining is a promising tool for the elucidation and optimization of crucial yet previously intractable aspects of bivalve hatchery production, genetic improvement, restocking, stock management, and conservation.

Keywords Neutral Red · Bivalves · Oocytes · Viability · Fecundity · Reproduction · Atresia

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Introduction

Bivalve capture fisheries and aquaculture account for approximately 15% of global annual seafood production, registering an eightfold increase over the bivalve production levels recorded in the 1970s (Wijsman et al. 2019). Although aquaculture is the major focus of bivalve production, fisheries management, restocking, conservation, and pollution monitoring are also important objectives (His et al. 1999; Gomez and Mingoa-Licuanan 2006; Arnold 2008; Joaquim et al. 2008; González-Wangüemert et al. 2018; Kreeger et al. 2018; Shantharam et al. 2019).

The need to assess oocyte quality for improved bivalve production has been recognized for decades (Utting and Millican 1997); however, this criterion has not yet been incorporated into broodstock genetic improvement efforts (Boudry 2009; Barros et al. 2018). Similarly, the assessment of fecundity and elucidation of early life stage mortality is, either directly or indirectly, at the heart of stock-recruitment analysis (Aoyama 1989; Koslow 1992; Jennings et al. 2001; Maunder and Thorson 2019), yet the evaluation of oocyte quality in spawns is a missing element in this process.

To date, assessments of oocyte quality have often been performed retrospectively, through fertilization rates, based on polar body or cell division observations (Uttig and Spencer 1991, Buttner and Weston 2010, Militz and Southgate 2021), analysis of chemical composition, and performance indicators (Caers et al. 1999, 2002; Massapina et al. 1999; Nevejan et al. 2003; Cannuel and Beninger 2005; Corporeau et al. 2012; García-Corona et al. 2018). Histochemical staining of specific oocyte components, in particular lipids, may also yield information on oocyte quality (Valdez-Ramirez et al. 2002). Lipid-specific stains such as Oil Red O and Sudan Black have been used to evaluate the quality of spawned oocytes (Gallager and Mann 1986; Gallager et al. 1986; Gómez-Robles et al. 2005; Angel-Dapa et al. 2010); however, the level of a biologically - important chemical component does not signify that the organism or cell is even alive (Moens and Beninger 2018).

The criterion of viability is surely the most fundamental of all oocyte quality indicators. The presence of atresic oocytes within the gonad reduces the realized fecundity (F_{real}) compared to the potential fecundity (F_{pot} – Jennings et al. 2001); in the case of marine bivalves, this has been estimated at approximately 50% of the total gamete volume over the course of the gametogenic cycle (Chérel and Beninger 2017, 2019; Chérel et al. 2020). It is not known what proportion, if any, of these atresic and other non-viable (dead) oocytes are spawned along with normal oocytes. Ideally, therefore, evaluation of spawn viability should yield information on the number of live, dead, and dying (atresic) oocytes.

A promising candidate for bivalve oocyte viability assessment is the Neutral Red (NR) stain. First reported as a vital stain by Ehrlich (1894), various NR protocols emerged as standard tests for cell vitality, beginning late in the twentieth century (Winckler 1974; Nemes et al. 1979; Hammond et al. 1980), usually in the context of tissue cultures or single cells subjected to various challenges (Borenfreund and Puerner 1984; Triglia et al. 1991; Lowe et al. 1992, 1995a, b; Babich and Borenfreund 1993; Weeks and Svendsen 1996; Chiba et al. 1998; Svendsen et al. 2004; Repetto et al. 2008; Aguirre-Martínez et al. 2013; Patetsini et al. 2013; Hu et al. 2015; Liu et al. 2018). The technique was used as early as 1972 by Dressel et al. to differentiate between live and dead plankton, and continues to be so used to the present (Crippen and Perrier 1974; Horvath and Lamberti 1999; Tang et al. 2006; Elliott and Tang 2009; Zetsche and Meysman 2012; Da Luz et al. 2016). It is thus somewhat surprising that it has not yet been employed for the same purpose in bivalve oocytes. Conversely, the same can

be said of the mortal stain Trypan Blue, widely used to detect dead cells in the biomedical fields (Wales 1959; Talbot and Chacon 1981; Kwok et al. 2004; Strober 2015; Rajab and Demer 2019).

Ideally, a spawned oocyte viability assessment should have the following characteristics:

- Specificity for live oocytes
- Low per-test cost
- Low equipment cost
- Simplicity – for routine use by hatchery workers
- Rapid execution and rapid results – so that the oocytes may be either used or disposed of immediately after obtention
- Low sample size (to preserve the utility of the obtained oocytes)
- Universality – for use with any bivalve species

The present study documents the use of Neutral Red as a vital stain, to collect the first known data on the viability of oocytes obtained by induced spawning or gonad stripping in four species of commercially - important bivalves: *Cerastoderma edule* (L), *Crassostrea gigas* Thunberg, *Mytilus edulis* L, and *Tapes philippinarum* (Adams and Reeve).

Material and methods

Sampling and gamete obtention

All bivalves were haphazardly collected in the Traict du Croisic, on the French Atlantic coast (47° 17′ 26″ N, 2° 30′ 16.2″ W). *Cerastoderma edule* and *T. philippinarum* were sampled on the mudflat at low tide, whereas *Crassostrea gigas* and *M. edulis* were sampled on adjacent rocky substrates. Sampling for *Cerastoderma edule* was performed on April 17 and May 2, 2019, because previous work had shown them to be spawning-competent at this early date in the gametogenesis season (Chérel and Beninger 2019). Only individuals >20 mm shell length (SL) were retained, corresponding to the size at which normal gamete production is achieved (Mejuto 1984; Pérez Camacho and Román 1984). The actual size range was 20.8–35.1 mm, and the corresponding shell age rings were 1–2.5 years.

Additional sampling was performed on 11 and 25 June 2019, at which time all four species were in advanced gametogenesis, and on 31 July 2020, when spawnings were known to occur. *M. edulis* specimens were all > 40 mm SL, *T. philippinarum* were all > 20 mm SL, and *Crassostrea gigas* were all > 20 mm SL; the latter to ensure that some males were sampled for spawning induction.

Spawning induction was repeatedly attempted for all four species, using various thermal shock–emersion regimes, as well as the addition of spermatozoa stripped from male individuals. Successful induction was obtained for at least 8 *Cerastoderma edule*, 3 *M. edulis*, and 1 *Crassostrea gigas* specimens. Experience showed that cockles spawned more readily in groups of 3–4 individuals, so it was not possible to ascertain the exact number of individuals that spawned (especially since release occurred in short "dribbles" of nearly transparent oocytes). Gamete stripping was used to obtain oocytes from all individuals for which spawning induction failed. Table 1 summarizes the numbers of individuals actually used for each staining assay. As *Cerastoderma edule* was the most oogenically advanced of the four species

Table 1 Number of females from which gametes were obtained by induced spawning or gonad stripping. *At least one of the spawning group per spawn

Species	Spawn	Stripping
Cerastoderma edule	8*	3
Mytilus edulis	3	1
Crassostrea gigas	1	3
Tapes philippinarum		5

studied in April and May 2019, spawning induction was attempted every day, up to 8 days following sampling (Table 2); specimens were kept in cold storage (emersion, 4 °C) between spawning attempts.

Staining procedure

Neutral Red was used as a vital stain, and for contrast, Trypan Blue was employed as a mortal stain. Exploratory work showed that the best staining results were obtained with Neutral Red at 5 mg ml^{-1}, and Trypan Blue at 2 mg ml^{-1} of filtered seawater. Although a large amount of undissolved Trypan Blue remained at this latter concentration, it was nonetheless necessary in order to obtain sufficient dissolved product capable of staining dead oocytes. Oocytes transferred to a microscope slide using a Pasteur pipette could be observed under an Olympus Provis AX70 optical microscope within 3 min of addition of a drop of NR prepared stain. TB staining required at least 1 h incubation time at room temperature prior to observation. Oocyte counts were performed for *Cerastoderma edule* at 40× on a haphazardly-chosen slide transect using Olympus cellSens Standard software; counting was stopped at 200 oocytes.

Table 2 *Cerastoderma edule* mean oocyte viability counts (%). *N*, number of females; *NR*, Neutral Red stained; *TB*, Trypan Blue stained

Sampling dates	Days in cold storage Spawning dates	N	NR		TB
			Normal Range	Atresic/abnormal Range	Dead Range
2/05/19	1	4	14.6	81.8	3.6
	03/05/19		19.4	23.0	7.4
17/04/19	2	3	30.0	65.6	4.3
	19/04/19		28.3	32.2	3.9
2/05/19	4	7	41.7	54.6	3.7
	06/05/19		59.5	48.5	14.3
	5	5	34.3	41.9	23.8
	07/05/19		42.1	39.5	32.2
17/04/19	6	3	66.4	23.7	9.9
	23/04/19		2.7	14.4	17.1
2/05/19	7	4	34.6	46.2	19.2
	09/05/19		79.8	47.1	32.7
	8	2	14.3	11.9	73.9
	10/05/19		12.0	2.6	12.0
Means of pooled counts			34.9	48.8	15.2
Range			79.8	79.1	78.5

Oocyte categories

In contrast to histological preparations, the whole mount preparations necessary for examination of spawned oocytes do not provide sufficient cellular detail to definitively identify atresic oocytes; therefore, all live oocytes with abnormal shapes, cytoplasmic shrinkage, and ruptured cell membranes were designated as "atresic/abnormal." The three oocyte viability categories were thus (1) "normal" (live, i.e., NR stained, normally shaped, no cytoplasmic shrinkage or membrane rupture), (2) "atresic/abnormal" (variably NR stained, rendering atresic and abnormal characteristics evident), and (3) "dead" (unstained, little or no cytoplasm, ruptured membranes).

Results

Efficient determination of all three oocyte viability categories was not possible using unstained whole mounts (Fig. 1 a). Neutral Red stained both normal and atresic/abnormal oocytes of all four species studied. Atresic/abnormal oocytes were readily recognized by their irregular shapes, cytoplasmic shrinkage (undetectable in unstained cells), and ruptured cell membranes (Figs. 1 b–d and 2 a, b), rendered easily visible by the Neutral Red stain. Prior to staining with Trypan Blue, dead oocytes were either not stained by NR, or so faintly stained that they were easily recognized (Figs. 1, 2, 3, and 4). Oocytes stained with Trypan Blue were assumed to be dead (Figs. 1 b, c, e, 2 b, c). Only NR-stained, spherical oocytes developed into larvae (Fig. 4). The initial divisions produced uniformly NR-stained cells, whereas at the first larval stage, most of the NR appeared to have relocated to the spherical cell nuclei (Fig. 4).

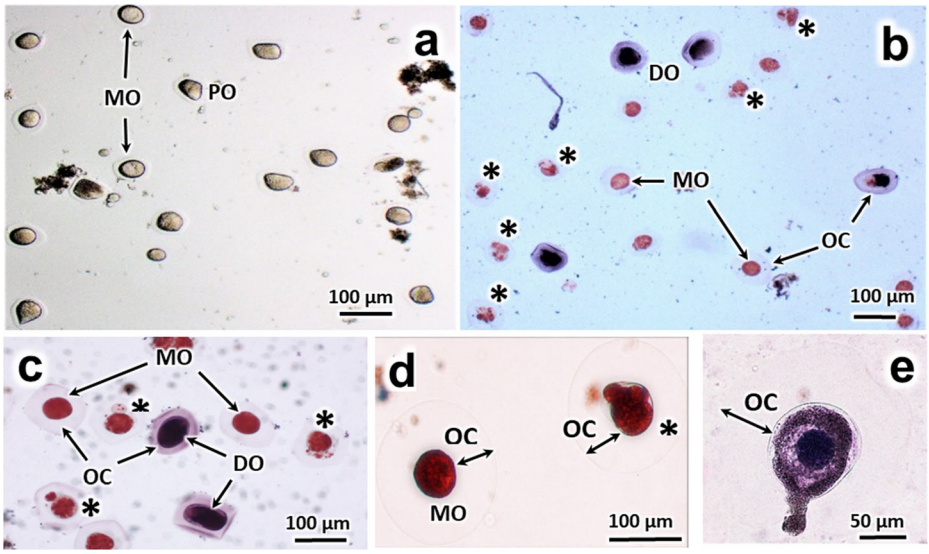

Fig. 1 *Cerastoderma edule* whole mounts. **a** Unstained, spawned oocytes. **b** Spawned oocytes double-stained with Neutral Red and Trypan Blue. DO, dead oocyte; MO, mature oocyte; OC, oocyte coat; the asterisk indicates atresic oocytes. **c**–**e** Details of oocytes from double-staining protocol: **c** details of mature, atresic, and dead oocytes; **d** detail of one mature and one atresic oocyte; note ooplasmic shrinkage; **e** detail of dead pedunculated oocyte

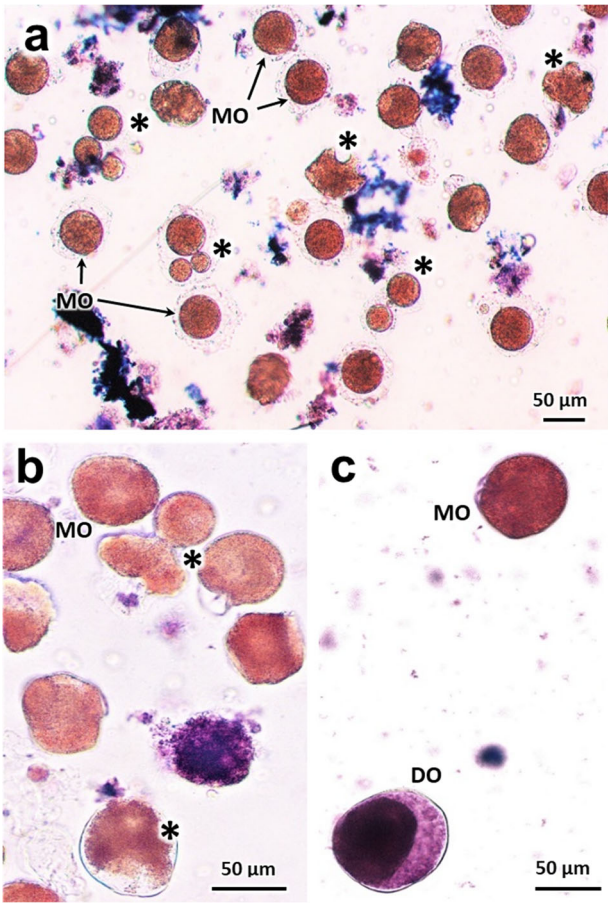

Fig. 2 *Mytilus edulis* spawned oocytes double-stained with RN and TB. DO, dead oocyte; MO, mature oocyte; the asterisk indicates atresic/abnormal oocytes

Count results for the three oocyte categories are presented in Table 2 for *Cerastoderma edule*, the only species for which more than 5 spawns were obtained (Beninger et al. 2012). The ranges of inter-individual variation were themselves quite varied, e.g., from 2.7 to 79.8% for normal oocytes, and 2.6 to 48.5% for atresic/abnormal oocytes. The corresponding means presented similar strong variations among the different sampling and spawning dates, from 14.3 to 66.4%, and 11.9 to 81.8%. The values for atresic/abnormal oocytes were generally superior to those of normal oocytes; dead oocytes were the smallest category except for the value obtained after 8 days of cold storage (73.9%). This being an observational study, no frequentist statistical tests were performed (Beninger et al. 2012; Beninger and Boldina 2018).

Discussion

Given the diversity of cell types in which a similar result has been obtained (Crippen and Perrier 1974; Triglia et al. 1991; Lowe et al. 1992, 1995a, b; Babich and Borenfreund 1993; Weeks and Svendsen 1996; Chiba et al. 1998; Horvath and Lamberti 1999; Svendsen et al.

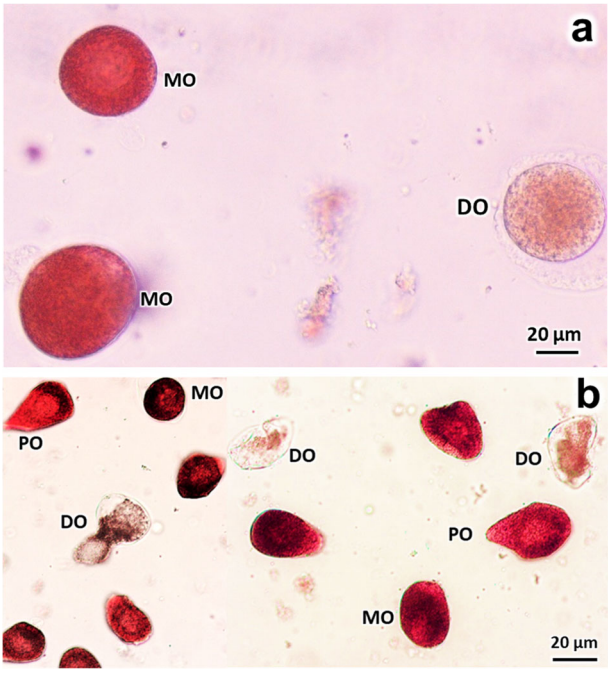

Fig. 3 **a** *Tapes philippinarum*–stripped oocytes stained with NR. MO, mature oocyte; DO, dead oocyte. **b** *Crassostrea gigas*–stripped oocytes stained with NR. PO, pedunculated oocyte. Most live oocytes are immature

2004; Tang et al. 2006; Repetto et al. 2008; Zetsche and Meysman 2012; Aguirre-Martínez et al. 2013; Patetsini et al. 2013; Da Luz et al. 2016; Liu et al. 2018), there was little reason to doubt that NR would also stain live bivalve oocytes. Yet beyond the necessity to verify this, it was important to determine how atresic/abnormal—"not dead yet"—oocytes would react with NR. As anticipated, these oocytes also stained to a variable degree with NR; yet the stain served to facilitate the detection of the abnormal cell shapes, retracted cytoplasm, and membrane rupture characteristic of such oocytes, and hence to allow their ready identification and quantification. Overall, contrasting use of Trypan Blue was deemed unnecessary in routine work, especially since it was difficult to use, due to its low solubility in seawater and lengthy staining time (introducing an oocyte mortality artefact); it is also comparatively expensive.

The mechanism of NR vital staining has been the object of a great deal of iterative conjecture, which has become unverified conventional wisdom since the procedure was first presented by Ehrlich in 1894 (Jacques 1969; Nemes et al. 1979; Hammond et al. 1980; Borenfreund and Puerner 1985; Borenfreund et al. 1988; Triglia et al. 1991; Weeks and Svendsen 1996; Svendsen et al. 2004; Repetto et al. 2008). Later studies proposed lysosomes as the "*Zellen Körnchen*" (cell granules) in which Ehrlich observed the stain to be concentrated; the micrographs of Lowe et al. (1992) and Patetsini et al. (2013) Lowe et al. (1992) and Patetsini et al. (2013) are among the rare publications which actually support this interpretation—although not all lysosomes of a given cell appear to have this property (Winckler 1974).

Notwithstanding the conjecture concerning the exact dynamics of NR within cells, it may be confidently assumed to cross the cell and lysosomal membranes (although the mechanism is not known); it is accumulated in the lysosomes due to a much slower re-diffusion to the

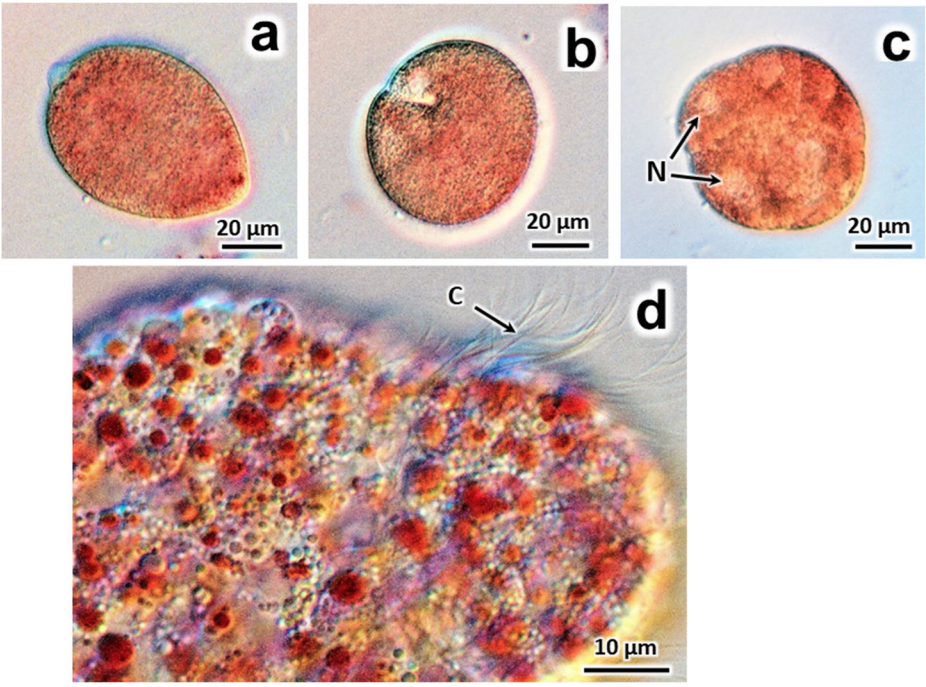

Fig. 4 *Crassostrea gigas* whole mounts. Early development of stripped, fertilized oocytes stained with NR. **a** Immediately after fertilization. **b** Initiation of first cytokinesis. **c** 8-cell stage, nuclei (N) clearly visible. **d** Second day after spawning and staining, early trochophore larva. Note NR stain now appears concentrated in spherical nuclei. C, cilia

cytoplasm. Re-diffusion to the cytoplasm and the external medium is accelerated by physical membrane damage, and possibly impairment of the lysosomal proton pump (Winckler 1974; Lowe et al. 1992; Patetsini et al. 2013). Our results show that after leaving the lysosome, NR appears to enter and be retained in the nuclei of *Crassostrea gigas* trochophore larvae. These observations confirm that the oocytes which took up NR were viable, and some of them developed into larvae.

Prior to the present study, it was not known whether the atresic oocytes, identified in the gonad by histology, were absorbed prior to spawning, or were incapable of being spawned, or if some or all of them appeared in the spawn. Our observations confirm that a large proportion of atresic/abnormal oocytes are spawned along with the normal oocytes. The high proportion of atresic and dead oocytes observed in the present study (30–85%) is entirely consistent with the previously published quantitative histological data (Chérel and Beninger 2017, 2019).

It is noteworthy that the animals used in the present study were sampled in the field, rather than being carefully conditioned in a hatchery environment. This may explain the very large discrepancy with the proportions of inviable oocytes in controlled hatchery spawnings, which are considered failures at >10% non-zygotes (Uttig and Spencer 1991, Buttner and Weston 2010, Militz and Southgate 2021). Taken together, these observations suggest that oocytes within broodstock may be particularly sensitive to adverse, or even normally-variable external conditions.

A high proportion of inviable post-fertilization stages is typical of high-fecundity species, including bivalves (Plough et al. 2016; Plough 2018); here we show that these high-fecundity species are also characterized by a high proportion of inviable pre-fertilization oocytes. The

atresia-fraught diminishing returns of many bivalves' high fecundity have been likened to a reproductive Red Queen dilemma: greater oocyte production does not translate to a commensurate increase in real fecundity (Chérel et al. 2020).

The very wide range of values for inviable oocytes from different individuals and time periods observed in the bivalve species studied here is also consistent with the Sweepstakes Reproductive Success (SRS) hypothesis for typical high-fecundity, type III survivorship marine species. Although at the population level, this strategy enables optimal exploitation of the rapidly changing environmental conditions of the nearshore marine environment, at the individual level it has many "losers" and few "winners" (Hedgecock and Pudovkin 2011). Further support for a strong genetic inviability–SRS component to the phenomenon of oocyte atresia comes from previous studies which showed that levels of atresia remain similar under widely different physical perturbation conditions (Chérel and Beninger 2017, 2019). Environmental aggression may contribute additional effects, as reviewed/shown in the gonad in Beninger (2017) and Smolarz et al. (2017), and demonstrated in the spawns of the present study with increasing spawner times in cold storage.

Conclusion

To our knowledge, the Neutral Red procedure outlined herein is the first and only technique which incorporates all of the essential performance criteria outlined above, allowing the rapid, easy detection of live, atresic/abnormal, and dead oocytes in spawns or gonad strippings. Its success in all four bivalve species suggests that it may be a universal tool for the evaluation of bivalve oocyte spawn quality. It can be used expeditiously for this purpose prior to hatchery fertilization and larval rearing, especially in the absence of prolonged, careful conditioning, and also as a phenotypic marker for genetic improvement of broodstock. Application to restocking is also important, since stock production estimates are heavily dependent on determinations of F_{real}, i.e., the number of *viable* oocytes produced (Aoyama 1989; Koslow 1992; Jennings et al. 2001; Maunder and Thorson 2019). Other important, overlapping applications include conservation and environmental monitoring.

The oocytes of many benthic marine invertebrates are similar in overall structure and composition (Adiyodi and Adiyodi 1983; Wourms 1987). Future research may therefore show NR vital staining to be a valuable tool in assessing oocyte viability in diverse marine taxa, including the ecologically- and commercially-important Anthozoa, Polychaeta, and Gastropoda.

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Data availability All files from this study are available from the authors upon reasonable request.

Declarations

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Conflict of interest The authors declare no competing interests.

References

Adiyodi K, Adiyodi R (1983) Reproductive biology of invertebrates. Vol. I: Oogenesis, oviposition, and oosorption. Wiley, New York

Aguirre-Martínez GV, Buratti S, Fabbri E, DelValls AT, Martín-Díaz ML (2013) Using lysosomal membrane stability of haemocytes in *Ruditapes philippinarum* as a biomarker of cellular stress to assess contamination by caffeine, ibuprofen, carbamazepine and novobiocin. J Environ Sci 25:1408–1418

Angel-Dapa MA, Rodríguez-Jaramillo C, Cáceres-Martínez CJ, Saucedo PE (2010) Changes in lipid content of oocytes of the penshell *Atrina maura* as a criterion of gamete development and quality: a study of histochemistry and digital image analysis. J Shellfish Res 29:407–413

Aoyama S (1989) The Mutu Bay scallop fisheries: scallop culture, stock enhancement, and resource management. In: Caddy JF (ed) Marine invertebrate fisheries: their assessment and management. Wiley, New York, pp 525–539

Arnold WS (2008) Application of larval release for restocking and stock enhancement of coastal marine bivalve populations. Rev Fish Sci 16:65–71

Babich B, Borenfreund E (1993) Applications of the neutral red cytotoxicity assay to risk assessment of aquatic contaminants: an overview. In: Environmental toxicology and risk assessment, W. Landis, J. Hughes and M. Lewis. ASTM International, West Conshohocken, PA, pp 215–219

Barros J, Velasco L, Winkler F (2018) Heritability, genetic correlations and genotype by environment interactions in productive traits of the Caribbean scallop, *Argopecten nucleus*(Mollusca: Bivalvia). Aquaculture 488:39–48

Beninger PG (2017) Caveat observator: the many faces of pre-spawning atresia in marine bivalve reproductive cycles. Mar Biol:164–163. https://doi.org/10.1007/s00227-017-3194-x

Beninger PG, Boldina I (2018) Quantitative considerations in mudflat ecology. In: Mudflat ecology. P.G. Beninger. Springer, Cham, Switzerland, pp 389–419

Beninger PG, Boldina I, Katsanevakis S (2012) Strengthening statistical usage in marine ecology. J Exp Mar Biol Ecol 426:97–108

Borenfreund E, Puerner JA (1984) A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). J Tissue Cult Meth 9:7–9

Borenfreund E, Puerner JA (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 24:119–124

Borenfreund E, Babich H, Martin-Alguacil N (1988) Comparisons of two in vitro cytotoxicity assays—the neutral red (NR) and tetrazolium MTT tests. Toxicol in vitro 2:1–6

Boudry P (2009) Genetic variation and selective breeding in hatchery-propagated molluscan shellfish. In: New technologies in aquaculture. G. Burnell and G. Allan. CRC Press-Woodhead Publishing Ltd, Boca Raton FL, pp 87–108

Buttner JK, Weston S (2010) Softshell clam culture: hatchery phase, broodstock care through seed production. Northeastern Regional Aquaculture Center Publication No. 202-2010, 12 pp

Caers M, Coutteau P, Cure K, Morales V, Gajardo G, Sorgeloos P (1999) The Chilean scallop *Argopecten purpuratus* (Lamarck, 1819): II. Manipulation of the fatty acid composition and lipid content of the eggs via lipid supplementation of the broodstock diet. Comp Biochem Physiol B: Biochem Mol Biol 123:97–103

Caers M, Utting S, Coutteau P et al (2002) Impact of the supplementation of a docosahexaenoic acid–rich emulsion on the reproductive output of oyster broodstock, *Crassostrea gigas*. Mar Biol 140:1157–1166

Cannuel R, Beninger PG (2005) Is oyster broodstock feeding always necessary? A study using oocyte quality predictors and validators in *Crassostrea gigas*. Aquat Liv Res 18:35–43

Chérel D, Beninger PG (2017) Oocyte atresia characteristics and effect on reproductive effort of Manila clam *Tapes philippinarum* (Adams and Reeve, 1850). J Shellfish Res 36:549–557

Chérel D, Beninger PG (2019) Oocyte atresia and its effect on reproductive effort of the common cockle *Cerastoderma edule* (Linneaus, 1758). J Shellfish Res 38:603–609. https://doi.org/10.2983/035.038.0300

Chérel D, Beninger PG, Le Pennec G (2020) Two enigmas may solve each other: the oocyte coat and atresia in the common cockle, *Cerastoderma edule* (Linnaeus, 1758). Mar Biol 167:104. https://doi.org/10.1007/s00227-020-03718-6

Chiba K, Kawakami K, Tohyama K (1998) Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. Toxicol in vitro 12:251–258

Corporeau C, Vanderplancke G, Boulais M, Suquet M, Quéré C, Boudry P, Huvet A, Madec S (2012) Proteomic identification of quality factors for oocytes in the Pacific oyster *Crassostrea gigas*. J Proteom 75:5554–5563

Crippen RW, Perrier J (1974) The use of neutral red and Evans blue for live-dead determinations of marine plankton (with comments on the use of rotenone for inhibition of grazing). Stain Technol 49:97–104

Da Luz DS, Da Silva DG, Souza MM et al (2016) Efficiency of Neutral Red, Evans Blue and MTT to assess viability of the freshwater microalgae *Desmodesmus communis* and *Ediastrum boryanum*. Phycol Res 64:56–60

Dressel DM, Heinle DR, Grote MC (1972) Vital staining to sort dead and live copepods 1, 2, 3. Chesapeake Sci 13:156–159
Ehrlich P (1894) Ueber Neutralrot. Allg med Zentral-Zeit 20:
Elliott DT, Tang KW (2009) Simple staining method for differentiating live and dead marine zooplankton in field samples. Limnol Oceanogr: Methods 7:585–594
Gallager SM, Mann R (1986) Growth and survival of larvae of *Mercenaria mercenaria* (L.) and *Crassostrea virginica* (Gmelin) relative to broodstock conditioning and lipid content of eggs. Aquaculture 56:105–121
Gallager SM, Mann R, Sasaki GC (1986) Lipid as an index of growth and viability in three species of bivalve larvae. Aquaculture 56:81–103
García-Corona JL, Rodríguez-Jaramillo C, Saucedo PE, López-Carvallo JA, Arcos-Ortega GF, Mazón-Suástegui JM (2018) Internal energy management associated with seasonal gonad development and oocyte quality in the Horsemussel *Modiolus capax* (Bivalvia; Mytilidae). J Shellfish Res 37:475–484
Gomez ED, Mingoa-Licuanan SS (2006) Achievements and lessons learned in restocking giant clams in the Philippines. Fish Res 80:46–52. https://doi.org/10.1016/j.fishres.2006.03.017
Gómez-Robles E, RodrIguez-Jaramillo C, Saucedo PE (2005) Digital image analysis of lipid and protein histochemical markers for easuring oocyte development and quality in pearl oyster *Pinctada mazatlanica* (Hanley, 1856). Journal of Shellfish Research 24:1197–1202. https://doi.org/10.2983/0730-8000(2005)24[1197:DIAOLA]2.0.CO;2
González-Wangüemert M, Basso L, Balau A, Costa J, Renault L, Serrão EA, Duarte CM, Hendriks IE (2018) Gene pool and connectivity patterns of *Pinna nobilis* in the Balearic Islands (Spain, Western Mediterranean Sea): implications for its conservation through restocking. Aquat Conserv: Mar Freshw Ecosyst 29:175–188. https://doi.org/10.1002/aqc.2976
Hammond M, Goodwin J, Dvorak H (1980) Quantitative measurements of neutral red uptake and excretion by mammalian cells. J Reticuloendothel Soc 27:337
Hedgecock D, Pudovkin AI (2011) Sweepstakes Reproductive Success in highly fecund marine fish and shellfish: a review and commentary. Bull Mar Sci 87:971–1002. https://doi.org/10.5343/bms.2010.1051
His E, Beiras R, Seaman M (1999) The assessment of marine pollution-bioassays with bivalve embryos and larvae. Adv Mar Biol 37:1–178
Horvath TG, Lamberti GA (1999) Mortality of zebra mussel, *Dreissena polymorpha*, veligers during downstream transport. Freshw biol 42:69–76
Hu W, Culloty S, Darmody G, Lynch S, Davenport J, Ramirez-Garcia S, Dawson K, Lynch I, Doyle H, Sheehan D (2015) Neutral red retention time assay in determination of toxicity of nanoparticles. Mar Environ Res 111:158–161. https://doi.org/10.1016/j.marenvres.2015.05.007
Jacques PJ (1969) Endocytosis. Lysosomes in biology and pathology 2:395–420
Jennings S, Kaiser MJ, Reynolds JD (2001) Marine fisheries ecology. Blackwell Scientific Publication
Joaquim S, Gaspar MB, Matias D, Ben-Hamadou R, Arnold WS (2008) Rebuilding viable spawner patches of the overfished *Spisula solida* (Mollusca: Bivalvia): a preliminary contribution to fishery sustainability. ICES J Mar Sci 65:60–64
Koslow JA (1992) Fecundity and the stock–recruitment relationship. Can J of Fish Aquat Sci 49:210–217
Kreeger DA, Gatenby CM, Bergstrom PW (2018) Restoration potential of several native species of Bivalve Molluscs for water quality improvement in Mid-Atlantic Watersheds. Journal Shellfish Res 37:1121. https://doi.org/10.2983/035.037.0524
Kwok AKH, Lai TYY, Li WWY, Yew DTW, Wong VWY (2004) Trypan blue- and indocyanine green-assisted epiretinal membrane surgery: clinical and histopathological studies. Eye 18:882–888. https://doi.org/10.1038/sj.eye.6701359
Liu X, Rodeheaver DP, White JC, Wright AM, Walker LM, Zhang F, Shannon S (2018) A comparison of in vitro cytotoxicity assays in medical device regulatory studies. Regul Toxicol Pharmacol 97:24–32. https://doi.org/10.1016/j.yrtph.2018.06.003
Lowe D, Moore M, Evans B (1992) Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Mar Ecol Prog Ser 91:135–140. https://doi.org/10.3354/meps091135
Lowe D, Fossato V, Depledge M (1995a) Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. Mar Ecol Prog Ser 129:189–196. https://doi.org/10.3354/meps129189
Lowe DM, Soverchia C, Moore MN (1995b) Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquat Toxicol 33:105–112. https://doi.org/10.1016/0166-445X(95)00015-V
Massapina C, Joaquim S, Matias D, Devauchelle N (1999) Oocyte and embryo quality in *Crassostrea gigas* (Portuguese strain) during a spawning period in Algarve, South Portugal. Aquat Living Resour 12:327–333
Maunder MN, Thorson JT (2019) Modeling temporal variation in recruitment in fisheries stock assessment: a review of theory and practice. Fish Res 217:71–86

Mejuto J (1984) Primeros datos sobre la dinámica de la población de *Cerastoderma edule* (L.) de la Ría do Pasaxe (NW de Galicia): estrategias de explotación. Actas IV Simp Iber Estud Bentos Mar 1:83–102
Militz TA, Southgate PC (2021) Chapter 3: Culture of giant clams. In: Shumway, S (ed) Molluscan shellfish aquaculture: a practical guide. 5M Books Ltd, Essex, pp 61-85
Moens T, Beninger PG (2018) Meiofauna: an inconspicuous but important player in mudflat ecology. In: Beninger PG (ed) Mudflat Ecology. Springer International Publishing, Cham, pp 91–147
Nemes Z, Dietz R, Lüth JB, Gomba S, Hackenthal E, Gross F (1979) The pharmacological relevance of vital staining with neutral red. Experientia 35:1475–1476. https://doi.org/10.1007/BF01962793
Nevejan N, Courtens V, Hauva M, Gajardo G, Sorgeloos P (2003) Effect of lipid emulsions on production and fatty acid composition of eggs of the scallop *Argopecten purpuratus*. Mar Biol 143:327–338
Patetsini E, Dimitriadis VK, Kaloyianni M (2013) Biomarkers in marine mussels, *Mytilus galloprovincialis*, exposed to environmentally relevant levels of the pesticides, chlorpyrifos and penoxsulam. Aquat Toxicol 126:338–345. https://doi.org/10.1016/j.aquatox.2012.09.009
Pérez Camacho A, Román G (1984) Crecimiento, reproducción, mortalidad y producción del berberecho *Cerastoderma edule* (L.), en la Ría de Arousa. Cuad Area Cienc Mariñ Sem Est Gal 499–507
Plough LV (2018) Fine-scale temporal analysis of genotype-dependent mortality at settlement in the Pacific oyster *Crassostrea gigas*. J Exp Mar Biol Ecol 501:90–98
Plough L, Shin G, Hedgecock D (2016) Genetic inviability is a major driver of type III survivorship in experimental families of a highly fecund marine bivalve. Mol Ecol 25:895–910
Rajab GZE-A, Demer JL (2019) Long-term results of surgical excision of conjunctival retention cyst using trypan blue with methylcellulose. Am J Ophthalmol Case Rep 14:28–31. https://doi.org/10.1016/j.ajoc.2019.01.010
Repetto G, del Peso A, Zurita JL (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3:1125–1131. https://doi.org/10.1038/nprot.2008.75
Shantharam AK, Padilla DK, Peterson BJ, Doall M, Lobue C, Webb A (2019) Macrofaunal community structure following the restocking of Northern Quahog (*Mercenaria mercenaria*) to Great South Bay, Long Island, NY. J Shelf Res 38:259–270. https://doi.org/10.2983/035.038.0206
Smolarz K, Hallmann A, Zabrzańska S, Pietrasik A (2017) Elevated gonadal atresia as biomarker of endocrine disruptors: field and experimental studies using *Mytilus trossulus* (L.) and 17-alpha ethinylestradiol (EE2). Mar Poll Bull 120:58–67. https://doi.org/10.1016/j.marpolbul.2017.04.007
Strober W (2015) Trypan Blue exclusion test of cell viability. Curr Protoc Immunol 111:A3.B.1-A3.B.3. doi: https://doi.org/10.1002/0471142735.ima03bs111
Svendsen C, Spurgeon DJ, Hankard PK, Weeks JM (2004) A review of lysosomal membrane stability measured by neutral red retention: is it a workable earthworm biomarker? Ecotoxicol Environ Safety 57:20–29. https://doi.org/10.1016/j.ecoenv.2003.08.009
Talbot P, Chacon RS (1981) A triple-stain technique for evaluating normal acrosome reactions of human sperm. J Exp Zool 215:201–208. https://doi.org/10.1002/jez.1402150210
Tang KW, Freund CS, Schweitzer CL (2006) Occurrence of copepod carcasses in the lower Chesapeake Bay and their decomposition by ambient microbes. Estuar Coast Shelf Sci 68:499–508
Triglia D, Braa SS, Yonan C, Naughton GK (1991) In vitro toxicity of various classes of test agents using the neutral red assay on a human three-dimensional physiologic skin model. In Vitro Cell Devel Biol-Animal 27:239–244
Uttig SD, Spencer BE (1991) The hatchery culture of bivalve mollusc larvae and juveniles. Laboratory Leaflet, Ministry of Agriculture, Fisheries and Food Fisheries Research, Lowestoft 68:31 pp
Utting SD, Millican P (1997) Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability. Aquaculture 155:45–54
Valdez-Ramirez ME, Donval A, Le Pennec M (2002) Ultrastructural and histochemical criteria for determining normality in mature oocytes of the Pacific oyster *Crassostrea gigas*. J Shellfish Res 21:707–714
Wales R (1959) The differential staining of human and dog spermatozoa. Aust J Exp Biol Med 37:433–439. https://doi.org/10.1038/icb.1959.44
Weeks JM, Svendsen C (1996) Neutral red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: A simple biomarker of exposure to soil copper. Environ Toxicol Chem 15:1801–1805. https://doi.org/10.1002/etc.5620151022
Wijsman JWM, Troost K, Fang J, Roncarati A (2019) Global production of marine bivalves. Trends and challenges. In: Smaal AC, Ferreira JG, Grant J et al (eds) Goods and services of marine bivalves. Springer International Publishing, Cham, pp 7–26
Winckler J (1974) Vitalfärbung von Lysosomen und anderen Zellorganellen der Ratte mit Neutralrot. Prog Histochem Cyto 6:III–89. https://doi.org/10.1016/S0079-6336(74)80001-X

Wourms J (1987) Oogenesis. In: Reproduction of marine invertebrates, Vol 9: General aspects, seeking unity in diversity. Blackwell Scientifc Publications, Palo Alto, pp 117–124
Zetsche EM, Meysman FJ (2012) Dead or alive? Viability assessment of micro-and mesoplankton. J Plankton Res 34:493–509

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