Genetics & Epigenetics in Life History and Reproduction: Oysters

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Introduction

Oysters represent a cosmopolitan molluscan taxa with a rich diversity of reproductive strategies that makes them a unique study system from a comparative physiology perspective. Oyster sex is greatly influenced by the environment and most species demonstrate varying degrees of hermaphroditism. In addition to having fascinating reproductive biology, oysters are ecologically and economically important marine invertebrates. They serve a critical ecological niche, serving as habitat and, as benthic filter feeders, contributing to the removal of excess organics, nutrients, and particulates. Additionally, several oysters species have gained culinary prominence, supporting fisheries and aquaculture industries. Commercial and recreational harvest of wild populations contributes to local economies and provides an important source of protein and nutrients. Aquaculture of bivalve species such as oysters has ancient origins, but has increased in recent decades. Today, shellfish aquaculture accounts for 76% of marine aquaculture production worldwide (FAO, 2012). The oyster that makes up the majority of the production and harvest worldwide is *Crassostrea gigas*, regionally referred to as the Pacific oyster (**Fig. 1(a) and (d)**). In addition to *Crassostrea*, there are several other families in the order *Ostreidae*, considered 'true oysters', including *Ostrea* and *Saccostrea*. *Pteroida* is another oyster order that represents the pearl oysters such as those in the genus *Pinctada*.

This article will cover what is known regarding sex determination and gametogenesis in oysters from a genetics and epigenetics perspective, with a focus on *Ostreidae*. Most of what is known comes from studies in *C. gigas*, as this species has received significant scientific attention given its high commodity value. This includes a genome sequence (Zhang *et al.*, 2012) that has contributed to understanding the molecular underpinnings of reproductive biology. Much of what is known about reproduction in oysters relates

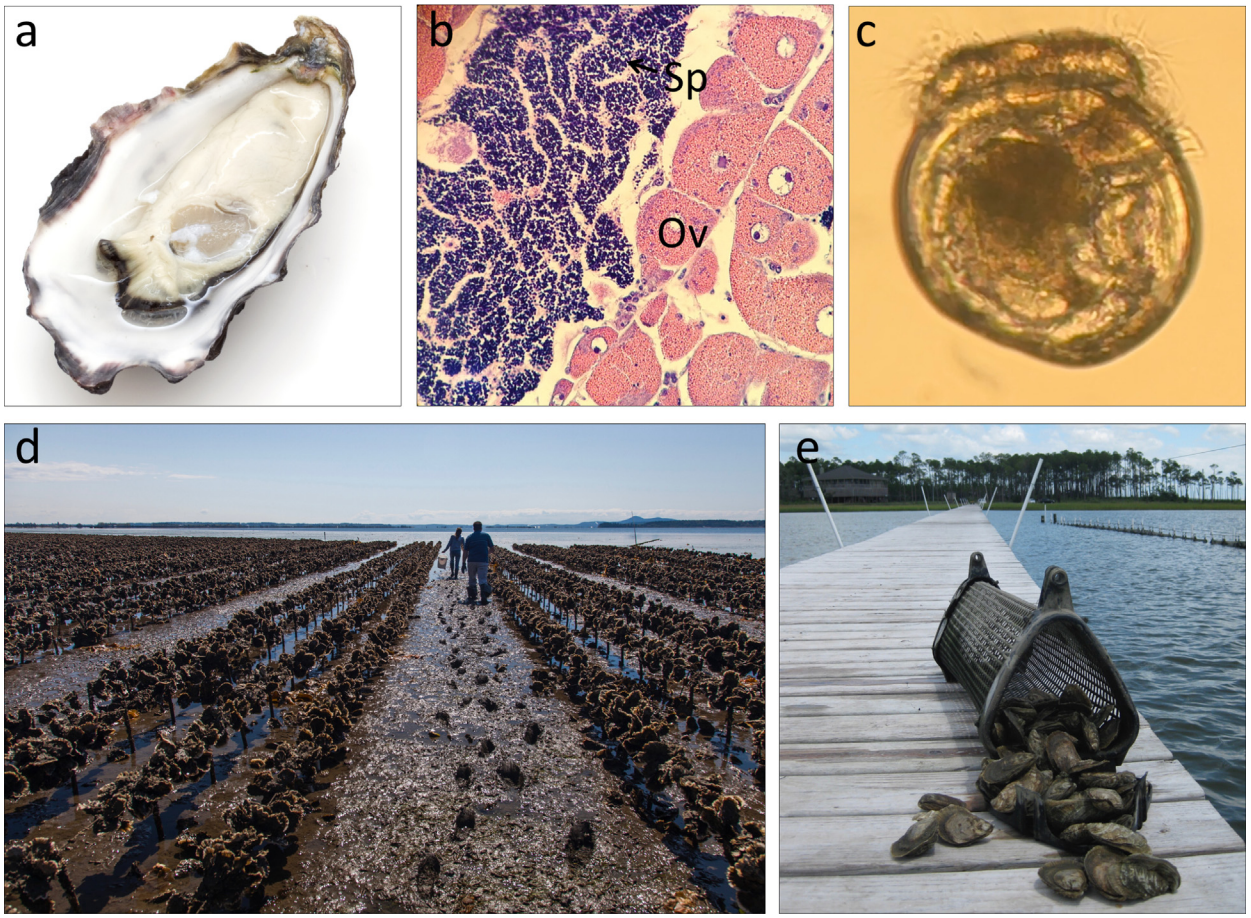

Fig. 1 Images of oyster life-stages and farming practices (a) adult *C. gigas*, (b) histological slide of a hermaphroditic *O. lurida* developing both male and female gametes, (c) *O. lurida* veliger larvae (planktonic stage), (d) *C. gigas* aquaculture in Washington, United States using long-lines, (e) *C. viginica* aquaculture in Mississippi, United States using off-bottom floating bags.

Encyclopedia of Reproduction, Second Edition, 2018, 736–742

to aquaculture and the desire to control gonad development. Before getting into molecular aspects, we will first provide a survey of life history strategies, paying particular attention to reproduction. This article concludes with perspectives on the role of epigenetic processes in oyster reproduction and future directions of research that could provide a mechanistic connection between environmental conditions and reproduction.

Life History Strategies

As juveniles and adults, oysters are sessile, benthic organisms inhabiting intertidal and subtidal marine and estuarine environments. Oysters, like many marine invertebrates, exhibit an r-selected reproductive strategy, meaning they produce a high number of 'cheap' offspring, but relatively few survive to maturity. While juvenile and adult oysters are sessile (**Fig. 1(a)**), early developmental stages are planktonic (**Fig. 1(c)**). Interestingly, from a comparative physiology perspective, various oyster genera within the order *Ostreidae* take different approaches to larval investment.

Oysters of the genera *Crassostrea* and *Saccostrea* are gonochoristic, alternate hermaphrodites with low but consistent frequency of simultaneous hermaphroditism (Coe, 1936). Some species, such as *C. gigas*, are protandric, meaning they mature first as males then as females in subsequent seasons. However, recent studies have reported that *C. gigas* is capable of changing sex within a single spawning season (Yasuoka and Yusa, 2016). Oysters of these genera are broadcast spawners. Broadcast spawners release male and female gametes into the water column where external fertilization occurs. The larvae that develop from external fertilization are consequently free-living, and remain in the water column consuming primarily phytoplankton for a number of weeks, depending on the species and environmental conditions.

Alternatively, flat oysters of the genus *Ostrea* are viviparous and female oysters of this genus brood their larvae. Oysters in the genus *Ostrea* exhibit rhythmic consecutive hermaphroditism, where they can flip back and forth maturing as males then females and back again during a single spawning season (Coe, 1943). As may be expected with this strategy, histological observations of the gonad in *Ostrea* species tend to show both male and female stages (**Fig. 1(b)**) though they spawn only as a single sex at a time. Mature male *Ostrea* species will release sperm balls into the seawater enabling uptake into the mantle cavity of the female where fertilization occurs. Females will brood larvae in the mantle cavity for approximately 1–2 weeks depending on the species and environmental conditions, then release the shelled larvae into the water column where they will be planktonic for an additional 1–2 weeks. Compared to the broadcast spawners, brooding oysters produce relatively larger, but fewer number of eggs and sperm. Regardless of genus, larval oysters will end their planktonic stage once they are competent to settle on suitable substrate (such as the shells of conspecifics) and will metamorphose into juvenile oysters, sometimes referred to as 'spat', at which time they begin a sessile existence.

Sex Determination

Sex determination remains poorly understood in oysters as in other bivalves. The mechanisms underlying the high plasticity of sex in oysters, particularly in *Ostrea* species that alternate sexuality within a single season, remain a topic for future work. Nevertheless, there is evidence in *Crassostrea* species to support a combination of genetic sex determination (GSD) where sex is established at fertilization, and environmental sex determination (ESD) where sex is determined after fertilization in response to environmental cues. The following sections will describe what is known about genetic and environmental influences on sex determination in oysters, primarily in *C. gigas*.

Genetic Sex Determination

Heteromorphic sex chromosomes have not been identified in any oyster species examined to date. A vast majority of the evidence for a genetic basis for sex determination in oysters come from sex ratio studies, where the sex ratios of offspring from known parents is measured. The first evidence for GSD in oysters came from a study with controlled crosses in *C. virginica*, where significant differences in sex ratios were observed between families sired by the same male (Haley, 1977). Subsequently, Guo *et al.* (1998) extended these findings by evaluating sex ratios in pair-mated *C. gigas* families and also reported significant family and parental effects on sex ratio. Based on these results, Guo *et al.* (1998) proposed a single-locus model of sex determination in *C. gigas* with a dominate male allele (M) and a protandric female allele (F), such that FM oysters are true males and FF individuals are protandric females that have the potential to sex-change. This model was not sufficient to explain the heterogeneity in sex among families generated by a single male and different female parents, thus Hedrick and Hedgecock (2010) proposed a 3-genotype model with fixed females (FF) fixed males (MM) and protandric females (FM), and included additional parameter 'f' in the model, which is the probability that FM individuals will mature as females.

The models of sex determination in *C. gigas* were derived with no knowledge of the sex determining genes involved with this process. Support for a single major sex determining locus comes from a genetic analysis (i.e., quantitative trait loci, or QTL analysis) that identified a single locus moderately associated with sex in *C. gigas* (Hubert and Hedgecock, 2004). However, additional studies would be needed to rule out the possibility that other loci were also involved. Studies investigating genes involved in sex-determination are limited in bivalves, but have been recently facilitated by technological advances in the genomics field. In

general, the approach has been to identify and characterize orthologs of canonical sex determining genes in oysters to determine if the gene expression profiles are consistent with a role in sex determination. Initial studies were successful in identifying the presence of canonical sex determining genes in oysters including *Dmrt1* an important upstream regulator of male sex determination in mammals and *FoxL2* a conserved gene in female sex determination (Dheilly *et al.*, 2012; Teaniniuraitemoana *et al.*, 2014). However the expression patterns of these genes were not consistent with sex determining functions in oysters, meaning they were either expressed in the gonad of both males and females or in somatic tissues. The most comprehensive study to date comes from Zhang *et al.* (2014), who used a comparative approach to identify *C. gigas* homologs of majors genes in sex determining pathway from both mammals and model invertebrates (*Drosophila melanogaster* and *Caenorhabditis elegans*). Here, they expanded the search of homologs in *C. gigas* to larger families of genes than had been previously investigated. They identified *Sox30 in C. gigas*, a homolog to the mammalian sex determining *Sry* gene, which had a pattern of expression consistent with a sex determining function. They also identified *Dsx* (a transcription factor similar to Dmrt1 that plays a role in male sex determination and differentiation mammals) and *FoxL2* (a transcription factor that plays a key role in female sex differentiation). Based on their findings, the authors proposed a model where *Sox30* is the major sex determining gene that is directly or indirectly linked with *Dsx* and *FoxL2*. This is the first time an *Sry*-like gene has been associated with sex determination outside of the vertebrates. Based on their findings (Zhang *et al.*, 2014), concluded oyster sex determination pathways are more similar to mammalian systems than invertebrates such as *D. melanogaster* and *C. elegans*. Additional work would be needed to follow up on this premise and also to determine how environmental factors influence sex determination.

Environmental Sex Determination

While a there is clearly a genetic component of sex determination, genetics alone does not control sex in oysters, as various exogenous factors have been reported to impact sex ratios of oyster populations. Temperature has a significant effect on sex ratios in oysters. Typically high temperatures are associated with femaleness (Fabioux *et al.*, 2005; Lango-Reynoso *et al.*, 2006), but for some species, such as *C. corteziensis*, males are predominant at high temperature (Chávez-Villalba *et al.*, 2008). Food availability also has the ability to skew sex ratios in many bivalve species including *C. gigas*, where it has been shown that high food availability promotes the development of females (Lango-Reynoso, 1999). Exogenous steroids, such as 17beta-estradiol, have also been reported to skew sex ratios toward females in *C. gigas* when administered early (but not late) in development (Mori and Nakamura, 1969). Many of these studies examine the influence of exogenous factors on adult oyster sex ratios, but very few have studied the effect of environmental exposure at initial gametogenesis. An exception is a controlled laboratory study in *C. gigas* spat (i.e., recently metamorphosed juveniles) where sex-ratios switched from female to male-biased between 18 and 28°C (Santerre *et al.*, 2013). Interestingly, although *C. gigas* are reported to be protandrous (maturing as males first) more than half of the oysters in this study first matured as females under normal rearing temperatures, suggesting other factors, such as food abundance, may also be important for sex determination.

Gametogenesis

Just as with sex determination, environmental conditions influence gametogenesis in oysters. Temperature is a primary factor associated with gametogenesis but food availability and photoperiod are also important. Because temperature has such a strong influence on gametogenesis, it is not surprising that there is a large amount of variation in the timing and extent of reproductive output across geographic areas, and specifically latitudes (Berthelin *et al.*, 2000; Enríquez-Díaz *et al.*, 2009). Although there is variation among oyster species and even among populations within the same species, it is common for initiation of gametogenesis to occur in winter followed by an active phase of gametogenesis in spring when water temperatures increase. Maturation and spawning typically occur in summer followed by a resorption and resting phase. However, in addition to environmental factors, various aspects of oyster reproduction including reproductive timing and output have been shown to be under genetic control (e.g., Lannan *et al.*, 1980). Although a genetic effect has been identified, the role of genes and hormones continue to be explored. The remainder of this section will provide an overview of gonad development, followed by insight into gene and steroid hormone control of gametogenesis in oysters.

The gonad of oysters is a transient tissue. In *C. gigas*, the germline is specified by maternal cytoplasmic determinants (preformation) through the 4d cell lineage and larvae possess putative primordial germ cells (Fabioux *et al.*, 2004). Primordial germ cells will eventually give rise to mature male or female gametes, and when it comes to oysters, frequently both within the same individual. At the initiation of gametogenesis, small clusters of stem cells are scattered in conjunctive tissue. Then germ cells divide by mitosis and gonial proliferation induces the expansion of tubules that invaginate the area surrounding the digestive system. These developing gonadal tubules grow at the expense of storage tissue and at the final stage of maturation, prior to spawning, gonads completely fill the gonadal tubules. Gametogenesis is considered a period of negative energy balance in oysters due to the high metabolic cost of gamete production and a concomitant loss of up to 60% body weight (Mathieu and Lubet, 1993). As early as 1936, it was suggested that a connection exists between metabolism and reproduction in oysters (Orton, 1936). It is likely that the variation observed in the timing and extent of gametogenesis in oysters is influenced by the inherent ability of the oyster to store and metabolize glycogen. Molecular approaches have given insight into the role of energy storage and mobilization in the reproductive cycle in oysters. For example, expression of genes related to glycogen metabolism show seasonal variation in association with the gametogenic cycle

(Bacca *et al.*, 2005). Samain *et al.* (2007) reported significant differences in expression of glucose metabolism genes in oyster strains that vary in the extent of reproductive investment.

A seminal study by Dheilly *et al.* (2012) that examined transcriptome variation throughout reproductive development in *C. gigas* found over 2000 genes differentially expressed over the course of maturation in males and females, shedding light on genes involved both in early sex differentiation as well as gametogenesis. Using a microarray platform to study the gonadal maturation process in males (spermatogenesis) and females (oogenesis), Dheilly *et al.* (2012) identified a suite of genes indicative of these respective processes. A number of candidate genes for sex differentiation were identified in early stage gonad samples including *dpy1*, a histone methyltransferase essential for hermaphroditism in *C. elegans* (Hsu *et al.*, 1995) in early male gonads and *FoxL2* a highly conserved female specific transcription factor in early female gonads. In mature male gonads, a number of genes involved in ubiquitination and proteasomal degradation of ubiquitinated proteins were uniquely expressed. In females, there was evidence of elevated cell cycle activity in maturing gonads and interestingly *Methyl-CpG binding domain protein 2* (*MBD2*) was found to be highly expressed in mature female gonads. *MBD2* is involved in binding methylated DNA and thus presumably involved in gene regulatory activity. Dheilly *et al.* (2012) suggested the high expression of *MBD2* in developing oocytes may indicate an epigenetic transfer of information.

In vertebrates, sex steroid hormones are a critical part of reproductive physiology and regulate a variety of reproductive activities including sex differentiation, maturation and estrous cycles. However, unlike vertebrates, the role of sex steroids in sex differentiation is controversial in bivalves. Although findings can be contradictory, a majority of the evidence suggests that sex steroids are important regulators of bivalve reproduction involved in gametogenesis, vitellogenesis and spawning. All of the major sex steroid groups including estrogens, androgens and progestins have been identified in bivalves. Sex steroids may also play a role in regulating the metabolism of glycogen, proteins and lipids in bivalves. For example, injections of estradiol have been shown to stimulate glycogenolysis by regulating activities of glucose-6-phosphate dehydrogenase in *C. gigas* (e.g., Mori *et al.*, 1972). Vitellogenesis, an essential part of oocyte maturation in oysters, can also be regulated by estradiol (Matsumoto *et al.*, 1997). These results indicate the steroids may accelerate the metabolic rate in the gonad providing more energy to the maturing gametes.

Triploidy in Oysters for Aquaculture

In bivalves, as in most animals, nutrients and energy are required for two major physiological processes, reproduction and growth. By reducing the development of the gonad, more resources can be used for somatic growth. This has been exploited in oyster aquaculture through the use of non-reproductive triploid oysters. Triploidy is the state of an organism or cell having three sets of chromosomes in each nucleus, and differs from the natural diploid (two sets of chromosomes) state found in most animal cells. Triploid individuals are commonly sterile because homologous chromosomes cannot pair during meiosis. The benefits of triploidy in oysters is that reproduction is often accompanied by the deterioration in taste and quality of the flesh as glycogen stores are used up, thus triploid oysters can be harvested year round as quality does not decline during summer months when oysters would normally be sexually mature. It has also been shown that triploid oysters grow faster than their diploid counterparts (Allen and Downing, 1986).

Triploidy was first induced in oyster aquaculture in the early 1980s using chemical manipulation of fertilized eggs (Stanley *et al.*, 1981). Specifically, the compound cytochalasin B was used to chemically inhibit extrusion of the second polar body in fertilized eggs, resulting in the retention of two sets of maternal chromosomes and one set of paternal chromosomes. Currently, triploid oysters are produced through crossing tetraploid and diploid oysters. In this process haploid eggs from diploid females are fertilized by diploid sperm from tetraploid males. The first tetraploid oysters were generated in *C. gigas* and were obtained by inhibiting the first meiotic division of eggs from triploid females. Although a majority of triploid oysters are sterile, some individuals are capable of producing gametes that, under tight control, can be used to produce tetraploids. Triploid induction does not guarantee sterility and there are numerous reports of slow growing gonads and abnormal gametogenesis or gonads that are actually fertile. Temperature is an important factor here as well, as triploid oysters show higher rates of maturation under higher temperatures (Normand *et al.*, 2008).

Epigenetics in Oysters

The plasticity of functional sexual morphology coupled with the clear relationship between environmental conditions and gametogenesis suggests an intriguing role of epigenetics in oyster sexual differentiation and reproductive development. Epigenetics refers to heritable processes that alter gene activity without changes to the underlying DNA sequence (Jablonka and Lamb, 2002). In this respect, heritable could be used in a mitotic sense, such as heritable between cell divisions, and in some cases also in a meiotic sense meaning being passed to the next generation through the germ cells. Common epigenetic marks include DNA methylation, histone modifications and non-coding RNAs. All of these marks have been shown to have important roles in mediating processes involved in reproductive biology, though primarily in mammalian systems. One interesting exception is the work of Navarro-Martín *et al.* (2011) where they demonstrated DNA methylation of the aromatase promoter is mechanistically involved in temperature dependent sex determination in European sea bass. Specifically, elevated temperature was associated with increased methylation of the aromatase promoter, which silenced the expression of this key enzyme responsible for conversion of testosterone

to estrogen, thus resulting in masculinization. It has also been shown in the half-smooth tongue sole (*Cynoglossus semilaevis*), a fish that exhibits both GSD and ESD, that exposure to high temperature early in development induces a 'pseudomale' phenotype (sex revered genetic female) that can be inherited at a high frequency in F1 offspring (Chen *et al.*, 2014). This phenotype is associated with an inherited pattern of DNA methylation on the sex chromosome derived from the pseudomale parent, implying a role for DNA methylation in maintaining this phenotype across generations (Shao *et al.*, 2014). These examples highlight ways in which DNA methylation may be playing a role in facilitating environmental responses to sex determination in species that, similar to oysters, exhibit a combination of GSD and ESD. The presumption remains that epigenetic processes are integral in the link between the environment and biological traits in oysters (**Fig. 2**), however, caution must be taken when extrapolating possible mechanisms to oysters as DNA methylation landscapes are strikingly different between vertebrates and invertebrates and thus functional roles could differ.

DNA methylation in oysters, similar to other invertebrates, is much lower than vertebrates. Whereas approximately 70%–80% of CG dinucleotides are methylated in vertebrate genomes (Bird and Taggart, 1980), only about 15% are methylated in oysters, where it is further limited in the genome to primarily gene bodies (Gavery and Roberts, 2013). Even considering this dramatic difference in pattern between vertebrates and invertebrates characterization of DNA methylation patterns in *C. gigas* indicate that this epigenetic mark has an important role in regulating gene expression (Gavery and Roberts, 2013). There are have not been any studies carried out to look specifically at the regulatory role of DNA methylation in oyster reproduction, but some studies have assessed DNA methylation pattern in gametes . A study by Olson and Roberts (2015) showed that DNA methylation patterns in sperm between related individuals were more similar than unrelated individuals. A recent study by Riviere *et al.* (2017), found dynamic temporal DNA methylation patterns across development from oocytes to spat.

While most of the studies examining epigenetics in oysters have targeted DNA methylation, there have been efforts to describe miRNA and histone modifications, though none have been specifically designed to determine their role in sex determination or differentiation. Certainly one of the most intriguing aspects of environmentally induced epigenetic changes is the potential for epigenetic information to be transferred meiotically from parent to offspring via gametes. One example of how this may be accomplished is through the transmission of histone modifications in mature gametes. It has been shown in both mammals and zebrafish that during spermatogenesis, when most histones are replaced with protamines, certain modified histones are non-randomly retained and tend to mark important early embryonic development genes, suggesting that these marks may have a role transferring epigenetic information to the embryo (Brykczynska *et al.*, 2010; Wu *et al.*, 2011). Histones are not replaced with protamines in bivalve sperm as they are in mammals (Eirín-López and Ausió, 2009). Rather, canonical histones are replaced with various sperm nuclear basic proteins that can be classified as either protamine-like type, histone-type or protamine-type (Ausio, 1986, Eirín-López and Ausió, 2009). It will require additional work to determine to what extent canonical histones and variants are retained in bivalve sperm, but this avenue of research could be very valuable toward a greater understanding of the potential transmission of epigenetic information from parent to offspring in bivalves. Histone modifications themselves remain largely understudied in shellfish, but it has been reported that bulk histone methylation levels and the expression of histone demethylases were responsive to temperature during development, suggesting a role for histone modifications in mediating the physiological responses of oysters to temperature (Fellous *et al.*, 2015). Because of the importance of temperature in sex

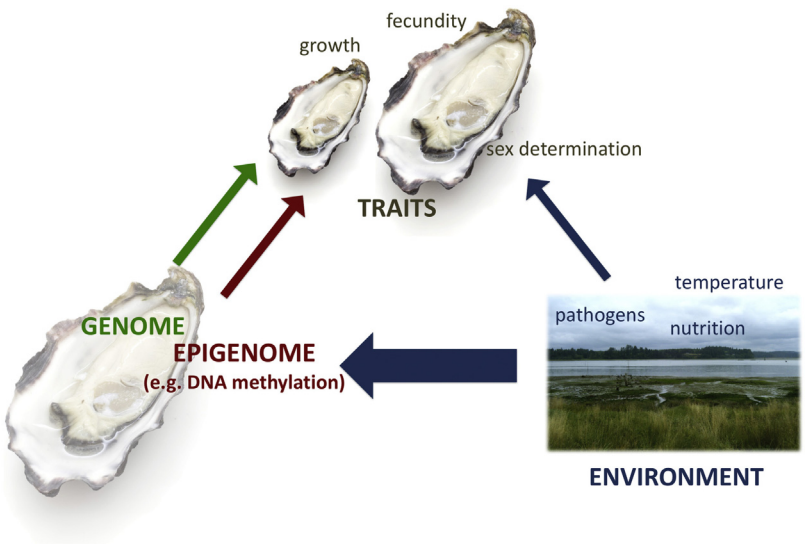

Fig. 2 An organism's genotype and the environment it encounters contribute to traits (phenotypes). Increasingly it's been shown that an organism's epigenetic information also contributes to phenotypes. Unlike the genome, the epigenome can be directly impacted by the environment, providing mechanisms to allow an organism to respond or adapt to external changes, and perhaps even transmit these induced traits to offspring.

determination and gametogenesis in oysters, additional studies on the role of histone modifications during these processes will be important.

Another important class of epigenetic marks that remain wholly uncharacterized in bivalve molluscs are regulatory microRNAs (miRNAs), though genes for miRNA biogenesis have been detected in available bivalves genomes (Rosani *et al.*, 2016). It has been shown that miRNAs exhibit sex-biased expression in the reproductive axis of vertebrates such as chicken (Bannister *et al.*, 2009) and Atlantic alibut (Bizuayehu *et al.*, 2012). Furthermore, miRNAs have been shown to play an important role during gonadogenesis in insects (e.g., Jin and Xie, 2007). Given the increasing evidence for the essential role of miRNAs in reproduction, it is likely that characterization of miRNA expression patterns during gametogenesis in oysters will provide insight into regulatory mechanisms of this process.

An often underappreciated taxa, oysters offer a unique system to explore the intersection of genetics and the environment and their respective roles in reproductive biology. As described here, there are still significant gaps in knowledge with regard to mechanistic underpinnings of sex determination and gonad development. Given that oysters are found worldwide in susceptible ecosystems, coupled with an expanding suite of genomic resources, there will likely be a focus on the oyster to help define the role of epigenetic phenomena in mediating genotype by environment interactions.

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