Breeding Focus 2021 - Improving Reproduction

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Preface

“Breeding Focus 2021 – Improving reproduction” is the fourth workshop in the series. The Breeding Focus series was developed to provide an opportunity for exchange between industry and research across a number of agricultural industry sectors. With this goal in mind, workshops have included presentations across multiple agriculturally relevant animal species to take participants outside their area of expertise and encourage them to think outside the box. Reproduction is a main driver for profitability and genetic gain. We will discuss existing knowledge, identify gaps and explore genetic and management strategies to improve reproduction further in multiple species.

Successful reproduction is a complex characteristic comprising the formation of reproductive cells, successful mating and fertilisation, embryonic and fetal growth and eventually a successful birthing event. In livestock species, reproduction traits have mostly low heritabilities, which makes it challenging to improve reproduction as part of a multiple trait breeding objective. The complexity arises not just from the cascade of processes required to result in successful reproduction, but the relevant traits are different in males and females and they are influenced through health and fitness, nutrition, climate and other environmental and management factors.

Challenges to the improvement of reproduction can vary widely for different species. For less domesticated species such as abalone, the ability to produce and reproduce the animals in captivity presents a major challenge. In bees, reproduction has not been given great attention and little research has been undertaken to understand the underlying genetics of drone and queen reproduction. However, in all industries reproduction is recognised as the basis for genetic and economic gain. It directly influences the selection intensity that can be applied. It also determines how many animals are not required for replacement and can be sold. In all industries, irrespective of the challenge, cost-effective and easy to measure phenotypes of reasonable heritability are central. New technologies and approaches enable the development of novel phenotypes for genetic improvement which will be combined with a growing amount of genomic data in livestock species and together these developments provide new and exciting opportunities to improve reproduction further.

We would like to thank everyone who has contributed to this event for their time and effort: the authors for their contributions to the book and presentations, the reviewers who all readily agreed to critique the manuscripts. We would like to express a special thanks to Kathy Dobos for her contributions into the organisation of this workshop and the publication. Thank you!

Susanne Hermesch and Sonja Dominik

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Reproductive challenges in abalone breeding

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Abstract

Abalone aquaculture in many countries has only recently closed the on-farm life cycle (managing all stages from production to reproduction) due to pressure on wild stocks and a move towards selective breeding for genetic improvement. Abalone breeding strategies rely on efficient maturation and spawning strategies to consistently produce healthy resilient animals. Maturation is controlled through temperature, photoperiod, and the availability of nutrients in the wild. These physical parameters are mimicked to promote synchronised maturation in aquaculture facilities through controlled photoperiod, controlled water temperatures and provision of macroalgae to supplement manufactured feeds. Spawning in the wild is subject to environmental pressures including temperature, tide, lunar cycle, photoperiod, food availability, and major storm events. The most common methods of inducing spawning in aquaculture systems are hydrogen peroxide and/or seawater UV irradiation coupled with heat shock which induces free radicals in the water. This activates prostaglandin and induces maturation of gametes and spawning. A variety of neuropeptides and hormones have been identified and characterised for their roles in gametogenesis, maturation, mating behaviour, and fertilisation. This has resulted in successful stimulation of gametogenesis by gonadotropin releasing hormone and spawning induction with egg laying hormone. As more of the hormonal cascade responsible for maturation and spawning is revealed alternative methods to prevent or induce reproductive behaviours have become possible. Despite the development of methods for artificial maturation and spawning induction, achieving synchronous spawning of male and female abalone at the desired time remains a major challenge for hatcheries. Precise control of spawning is critical for many advanced technologies, including the production of selected family lines, generation of hybrids and triploids, and for research programs. Applied solutions are under development to control maturation and spawning as well as develop the tools to measure environmental risk to abalone reproduction. To date, reproductive disruption strategies have focused on triploids to induce sterility, interspecific hybridisation, and ploidy. In contrast, the role of pollution in endocrine disruption has been demonstrated in abalone having adverse effects on reproduction and health.

Why improve reproduction in abalone?

Abalone species are found in both temperature and tropical climates throughout the world. Many temperate species grow to a larger size than tropical species and are highly valued for their palatable foot muscle (Hahn 1989; Jarayabhand and Paphavasit 1996). Domestication of aquaculture species enables increased production to meet market demand, enhanced disease and environmental tolerance, decreased predation risk, and recovery of threatened and endangered species (Ikhwanuddin and Abol-Munafi 2016). Domestication of livestock species such as goats, pigs, cattle and sheep occurred more than ten thousand years ago and poultry (chickens) around five thousand years ago (Larson *et al.* 2014). In contrast, abalone domestication first began in the 1960s in Japan before spreading around the world (Hahn 1989). Until recently abalone aquaculture production has predominately relied on annual spawning of wild broodstock (mature animals of breeding age and condition) to produce hatchery stock. The decline of many wild abalone fisheries due to poaching, overfishing and environmental disturbances have resulted in many aquaculture facilities managing all stages from production to reproduction in captivity (closing the on-farm production cycle) (Elliott *et al.* 2000).

Control of animal husbandry, including nutrition, water quality, and tank design, throughout the life cycle supports the closure of the production cycle on farm. Optimal abalone nutrition is supported through macroalgae or manufactured feeds. Manufactured feeds are often supplemented with the inclusion of live algae such as *Ulva* spp., *Gracilaria* spp., *Undaria* sp. and *Laminaria* sp. is reported to improve the health, reproductive condition and product quality of land-based abalone (Uki 1981; Bansemer *et al.* 2016). In addition, land-based systems often utilise shading, hides and dark tunnels to assist this nocturnal species to thrive in aquaculture (Buss *et al.* 2015). Water quality is particularly critical as pollution from pharmaceuticals (sewage), heavy metals (industry) and agricultural chemicals (including run-off) may result in endocrine disruption for this species (Langston 2020). In molluscan species this can impact growth, reproductive success, nutrition, and attachment to the substrate (Langston 2020). Reproductive success and fecundity are the cornerstones of a successful abalone aquaculture industry, whether for farm restocking or genetic improvement programs to increase production.

In contrast, elimination of maturation and spawning in general farm stock can be desirable in some cases. Reproduction is energetically demanding, and non-mature abalone display increased stress resilience and disease resistance compared to reproductively active animals (Najmudeen 2007; Travers *et al.* 2008). Maturing abalone are also a risk during live transport; not only is their physiology more fragile, but the stress involved in transport and holding of live animals may induce spawning, which leads to increased mortality during transport (Botwright *et al.* 2014). For individual breeders, however, elimination of maturation also provides the opportunity to protect the intellectual property of their elite selectively bred animals. Further, prevention of maturation safeguards the wild abalone fisheries, as accidental escapees into local populations are unable to influence the genetic diversity of local populations.

Despite the development of methods for artificial maturation and spawning induction, achieving synchronous spawning of male and female abalone at the desired time remains a major challenge for hatcheries, with only approximately 40% of broodstock expected to spawn (Elliott *et al.* 2000; Ritar and Elliott 2004; Al-Rashdi and Iwao 2008; Kim *et al.* 2018). Precise control of spawning is critical for many advanced technologies, including the production of selected family lines, generation of hybrids and triploids, and for research programs (Elliott *et al.* 2000), therefore development of efficient techniques will provide significant benefits to the industry.

Breeding for genetic improvement

Many farms in developed countries now utilise closed life cycle breeding due to declining wild stocks and/or a move towards genetic improvement programs (Elliott *et al.* 2000). Breeding programs generally target commercially important traits such as growth, disease resistance and stress tolerance as well as favourable marketable characteristics including growth, colour, flesh quality and quantity and behavioural characteristics amenable to aquaculture (Elliott *et al.* 2000; Hollenbeck and Johnston 2018). Breeding from select elite individuals rather than a group of abalone is required to achieve the greatest gains. For example, in molluscs growth rate and disease resistance may be improved by 10% and 15% per generation respectively (Hollenbeck and Johnston 2018). In contrast, some breeding programs are directed towards the recovery of wild stocks, particularly for endangered species, and therefore have different genetic requirements to farmed abalone (Rogers-Bennett *et al.* 2016). Nonetheless, both types of breeding programs rely on the use of mass selected, or genetically selected, broodstock that are primed for synchronised spawning on demand. A well-designed genetic improvement program whether individual or family-based ensures the continued viability of the abalone industry, or wild stocks, by reducing inbreeding (Elliott *et al.* 2000; Hollenbeck and Johnston 2018). A challenge for the industry is the inability to select an elite individual for spawning with 100% confidence of success, unlike other aquatic species, such as Atlantic salmon (*Salmo salar*), where synchronised spermiation and ovulation can be induced with commercially available analogues of gonadotropin releasing hormone enabling both sexes to be strip spawned concurrently to facilitate the mixing of gametes from specific individuals (Taranger *et al.* 2003).

Major challenges to controlled reproduction

Maturation

Temperature, photoperiod and availability of nutrients are the primary factors that regulate reproductive cycles in marine invertebrates including molluscs such as abalone (Giese 1959). Temperate abalone species only have one or two reproductive seasons per year, whereas tropical species may reproduce multiple times (Shepherd and Laws 1974; McShane and Naylor 1996; Jebreen *et al.* 2000). In abalone the gonad develops around the conical appendage of the digestive gland, and its development can be monitored utilising the visual gonad index (VGI) range, from 0-4 (Kikuchi and Uki 1974a). The VGI is a scoring system wherein “0” is assigned to a flat gonad and “3” denotes a swollen-round conical appendage. It was first developed to predict ripeness in *Haliotis discus hannai* where a VGI of 3 was associated with a high propensity to spawn (Kikuchi and Uki 1974a). Males and females are identified by the colour of the gametes visible through the thin outer membrane of the gonads. Male gonads are generally a creamy, white colour, while females are highly variable across the visible spectrum including, but not limited to, green, purple and blue depending on the species.

From a biological perspective, gonad conditioning is crucial for spawning success as formation of reproductive cells (gametogenesis) and deposition of yolk in the oocyte (vitellogenesis) take place at this stage (Hahn 1994; Grubert and Ritar 2005). Gonad maturation is controlled by an effective accumulation of temperature, measured in degree days (°C-days), which varies depending on the species (Kikuchi and Uki 1974a; Uki and Kikuchi 1984; Hahn 1994; Grubert and Ritar 2005). Therefore, the conditioning of stock is the first essential step to be mastered for a successful breeding program. There are few recirculated aquaculture systems in use within the abalone aquaculture industry, as such the incoming water temperature for broodstock holding systems is subject to the seasonal variation of local water temperatures, enabling the accumulation of degree days for gonad maturation. In addition, abalone are nocturnal with increased feeding and activity between dusk and midnight (Uki and Kikuchi 1984; Buss *et al.* 2015). Therefore, it is no surprise that gamete release also coincides with changes in photoperiod, ergo pre-conditioning abalone to a set photoperiod (12 h light:12 h dark) in the weeks leading up to spawning induction can assist to foster timely spawning in abalone aquaculture facilities. Finally, the move towards manufactured diets in many land-based facilities appears to leave a deficit in broodstock maturation of gonad and subsequent spawning activities for many abalone species. The inclusion of fresh algae, particularly *Undaria* sp. and *Laminaria* sp., during maturation improves gamete quality and fertilization success (Uki 1981).

All abalone exhibit the characteristic of re-conditioning gonad throughout the spawning season if temperature and nutrient availability are favourable. However, there is a risk that continuous re-conditioning with repeated spawning can lead to mortality. For example, summer mortality occurs as a result of warm water temperature spikes in summer and leads to the mass mortality of many ecologically and economically molluscs including abalone (Shiel *et al.* 2017). During summer, higher water temperature causes chemical changes in the water quality, such as salinity, pH, dissolved oxygen, elevated ammonia and nutritional factors (Vandepeer 2006). When abalone are under these altered external stimuli, spawning often occurs (Jobling *et al.* 2004; Mendoza-Porras, Botwright, *et al.* 2017), which is energetically expensive. If this occurs five or six times in the peak of summer, the abalone become exhausted from repeated re-conditioning of gonad, their immune system is weakened, and opportunistic pathogens may cause mortalities (Travers *et al.* 2008). There have been reports of summer mortality costing the aquaculture industry up to US$11.5 million in some countries (Chang *et al.* 2005).

Spawning

Abalone are broadcast spawners and rely on external environmental cues to synchronise the release of gametes. Gametes are expelled into the ocean where hydrodynamics and chemotaxis facilitate gamete recognition (Riffell *et al.* 2004; Himes *et al.* 2011; Zimmer and Riffell 2011). The cues are varied across species and include temperature, tide, lunar cycle, photoperiod, food availability, and major storm events, often in combination (Shepherd and Laws 1974; Uki and Kikuchi 1984; Counihan *et al.* 2001; Onitsuka *et al.* 2007). The exact requirements for natural spawning are difficult to determine, and are known to differ even between populations of the same species in different geographical locations (Uki and Kikuchi 1984; Counihan *et al.* 2001). Even if well understood for a particular species, these natural spawning cues are difficult or impossible to replicate in a hatchery setting, therefore achieving spawning on demand is challenging. This is problematic as farms may require broodstock conditioning and spawning out of their natural spawning season to maximise production, or if problems arise in obtaining enough offspring.

Traditionally, hatcheries have utilised a number of methods to artificially induce spawning, including desiccation, thermal shock, or the treatment of seawater with hydrogen peroxide or ozone-generating UV lamps. The most common methods are addition of hydrogen peroxide (~0.25-5 mM) with pH increase to 9.1 (Morse *et al.* 1977; Tanaka 1978) and/or seawater UV irradiation (50-253 nm) coupled with thermal shock above the biological optima for the species (Kikuchi and Uki 1974b). These methods exploit the stimulation of prostaglandin by the presence of free radicals in seawater, resulting in in the maturation of gametes and spawning (Morse *et al.* 1977); however the effectiveness of the method varies within and across abalone species (Kabir *et al.* 2001).

Reproduction in molluscs is at least partially controlled by various neuropeptides and hormones involved in gametogenesis, mating behaviour, and fertilization, many of which are widely conserved within molluscs or animals more broadly (Cummins *et al.* 2008, 2010, 2011; Morishita *et al.* 2010; York, Cummins, Degnan, *et al.* 2012; Giusti *et al.* 2013; Palmer *et al.* 2013). These induce a variety of physiological functions, including growth, contraction required for spawning, mating behaviours and sexual reversion (Table 1). Peptides of the FMRFa family, including several FMRF amide-related peptides (FaRPs), FMRFa-like peptides (FLPs), and amidated tetrapeptides (APGWa), act as cardio-excitatory molecules, neurotransmitters, neuromodulators and muscle relaxants (Croll and Van Minnen 1992; Fan *et al.* 1997; Worster 1998; Ohtani *et al.* 2002; Geoffroy *et al.* 2005; Panasophonkul *et al.* 2009; Cummins *et al.* 2011). In the sea-hare *Aplysia californica*, the well-known molluscan peptides FMRFa, FLRFa, and APGWa, were identified in a 430-5000 Da range neuropeptide mapping experiment (Li *et al.* 1998), and a recent study reported ganglion-specific expression and spawning influence of these peptides in *H. discus hannai* (Kim *et al.* 2018).

Table 1. Summary of peptides and their physiological action in molluscs. Modified from Morishita et al. (2010) *and enriched from York et al.* (2012).

Name	Sequence	Role	Species
APGWa	APGWa, TPGWa, KPGWa	Relax sperm ducts, reproduction antagonist	*Crassostrea gigas* *Thais clavigera*
CDCH-I	LSITNDLRAIADSYLYDQHKLRERQEENLRRRFLELa	Ovulation, control	*Lymnaea stagnalis*
CDCH-II	SITNDLRAIADSYLYDQHKLREQQEENLRRRFYELSLRPYPDNL	Shell turning	*L. stagnalis*
ELH	ISINQDLKAITDMLLTEQIRERQRYLADLRQRLLEKa	Egg-laying	*Aplysia californica*
aELH	LSITNDLRAIADSYLYDQHKLRERQEENLRRRFLRL	Spawning	*Haliotis rubra*
ERH	ISIVSLFKAITDMLLTKQIYANYFSTPRLRFYPI	Egg-releasing	*A. californica*
Califin A (L)	ISINQDLKAITDMLLTEQIQARRRCLDALRQRLLDLa	Neural modulation	*A. californica*
Califin A (S)	DSDVSLFNGDLLPNGRCS	Neural modulation	*A. californica*
α-CDCP	EPRLRFHDV	Egg-laying	*A. californica*
α-BCP	APRLRFYSL	Egg-laying	*A. californica*
TEP-1	KSCGKWAIHACWGGNa	Contractions penial	*T. clavigera*
TEP-2	KSYGKWAMHACWGGNa	complex	*T. clavigera*
FMRFa	FMRFa	Myoactive	*Fusinus perplexus ferrugineus*
Has-SLP	FSCDPECFSYLDGPFCISNGSVVCDNCLRDRMLCEDMSLTSKDCSAPCD	Growth	*H. asinina*
Has-HGAP	MKVLCILLVVVGTALSREVGKVARRDEDFVCPPQEESFGYMSFCAWDPCDIFADQPCADIPEAVCKFDACPNHACIPMFFVDKQWVECHDLVYRDK	Growth	*H. asinina*
FVa	GAPRFVa	Increase K+ current in pleural sensory neurons	*Aplysia* spp.
Novel peptide	HF (FH) FYGPYDVFQRDVa	Cease reproduction depending on age	*L. stagnalis*
F(X)RIa	ASSFVRIa	Unknown	Unspecified

Applied solutions

Maturation control

Control of maturation in abalone is currently limited to specific environmental parameters in aquaculture facilities. Some facilities with breeding programs have advanced the control of abalone maturation with the use of photoperiod and temperature-controlled facilities including heating the room, or incoming water, to improve reliability of gonad conditioning of elite individuals. However, there is also a drive to prevent maturation to improve resilience, protect wild fisheries from cultured escapees, protect intellectual property of elite breeding lines and retain live product quality by preventing spawning in response to stress. To date several hormones, and more recently peptides, involved in gonad maturation and neuronal control have been identified in molluscs (Figure 1). Injection of gonadotropin-releasing like hormone (GnRH-like) has been shown to stimulate gonad development and partial spawning in *Haliotis asinina* (Nuurai *et al.* 2010). Later studies by the same group showed that synthetic GnRH together with buserelin stimulate oogonia and oocyte proliferation in *H. asinina* (Nuurai *et al.* 2016). The role of GnRH in gonad maturation in abalone has also been verified in *H. discus hannai* (Sharker *et al.* 2020). In other molluscs, such as Pacific oyster *Crassotrea gigas,* injection of GnRH orthologs promotes DNA synthesis in gonial cells, whilst in *Mytilus edulis* mammalian GnRHs stimulated mitotic activity in mantle cells (Pazos and Mathieu 1999).

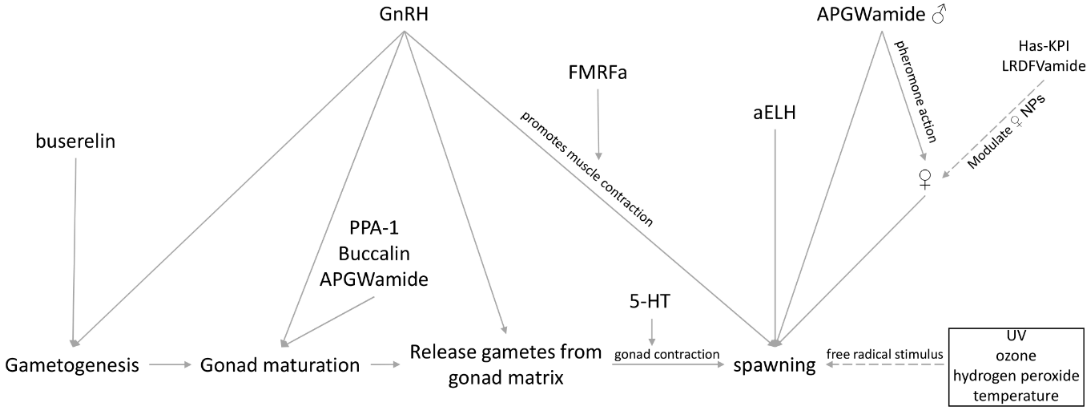

Figure 1. Diagram summarising current knowledge of hormones and neuropeptides involved in abalone maturation and spawning. GnRH – gonadotropin releasing hormone; PPA-1 – pedal peptide A-1; 5-HT – serotonin; aELH – abalone ELH; Has-KPI – Haliotis asinina Kazal-type protease inhibitor; NPs – neuropeptides.

Several peptides have been reported to influence neuronal control of maturation in abalone (Table 2) (Kim, Markkandan, *et al.* 2019; Botwright *et al.* 2019). Interestingly, except for pedal peptide A-1, most peptides reported in the literature produce inconsistent results between abalone species studied thus far. Pedal peptides have been reported for many molluscs including *A. californica*, *Lottia gigantia* and *Sepia officinalis* (Lloyd and Connolly 1989; Veenstra 2010; Zatylny-Gaudin *et al.* 2015), and are found to have roles in maturation as well as muscle contraction (Hall and Lloyd 1990). APGWamide and buccalin have also been reported to have roles in maturation in abalone and oysters (Chansela *et al.* 2008; York, Cummins, Degnan, *et al.* 2012; In *et al.* 2016). While synthetic peptides offer options to control maturation, more easily applied options include changing manufactured feed formulations to avoid ingredients that induce hormonal responses that promote gonad maturation such as dietary soya (Wu *et al.* 2019). Other alternative technologies such as triploidy, mimetics, RNAi, or gene editing technologies may offer more reliable or efficient methods for the elimination of maturation for farm-based life cycles. On-going research and public consultation will inform how effective and socially acceptable these strategies are for the aquaculture industry in the future.

Table 2. Haliotis spp. neuropeptides upregulated in response to maturation

Neuropeptide family	Peptide sequence	Species	Reference
APGWamide	TLDILEDYT	*H. laevigata* *H. asinina* *H. rubra*	Botwright *et al.* (2019) Chansela *et al.* (2008) York et al. (2012)
Enterin-1	LVNPFVYYALGKNKNSGNTP	*H. laevigata*	Botwright *et al.* (2019)
FMRFamide	AGEDGEEFEDDDEGLEAD	*H. discus hannai*	Kim *et al.* (2019)
	FMRFGKSGEEE	*H. discus hannai*	Kim *et al.* (2019)
	SGDDEKRFMRFamide	*H. discus hannai*	Kim *et al.* (2019)
LFRFamide	NFHWGRETEE	*H. discus hannai*	Kim *et al.* (2019)
MAP-5	RSLSLDDTYWVDNVD	*H. discus hannai*	Kim *et al.* (2019)
Pedal peptide A-1	SFDSINKGSGLSGFM	*H. discus hannai*	Kim *et al.* (2019)
	PLESIAAGRDLSGFV	*H. discus hannai*	Kim *et al.* (2019)
	PFDSIAAGSDLSGFA	*H. laevigata*	Botwright *et al.* (2019)
	PFDSISGSSAFSDFA	*H. discus hannai*	Kim *et al.* (2019)
	PFDSIASGGGMAGFA	*H. discus hannai* *H. laevigata*	Kim *et al.* (2019) Botwright *et al.* (2019)
	PLDSISAGSGLSGFA	*H. laevigata*	Botwright *et al.* (2019)
Pedal peptide A-3	APFDPLANELIE	*H. laevigata*	Botwright *et al.* (2019)
	PFDPLASGLI	*H. laevigata*	Botwright *et al.* (2019)

Spawning induction

The mechanisms that regulate spawning in abalone are not well known. Understanding which molecules are directly involved in spawning is key to improving spawning rates, and as knowledge of the molecular cascade that controls spawning in abalone and other molluscs grow, so too does the opportunity to apply advanced technologies to the problem of hatchery-based spawning induction. Within abalone, prostaglandin, 5-hydroxytryptamine (5-HT, or serotonin), dopamine, APGWamide, and egg-laying hormone (ELH) have shown potential for use as spawning inducers when injected into mature individuals (see Table 3), however the numbers of individuals used in each experiment tend to be small, and responses to injection of these hormones appear to be species-specific.

Despite the variability in response, a number of these hormones show promise for spawning induction. ELH (Arch and Smock 1977; Cummins and Hanna 2004) is a neuropeptide that promotes egg deposition, sexual maturation, mating behaviour and spawning in the molluscs *A. californica* and *L. stagnalis* (Roubos *et al.* 1981; Goldschmeding *et al.* 1982; Scheller *et al.* 1982; Geraerts *et al.* 1984; Bernheim and Mayeri 1995). ELH is actually encoded by a gene family, with each gene encoding a preprohormone that is cleaved after synthesis to produce a number of different peptides, and each of these peptides can influence different behaviours (reviewed in Cummins and Hanna (2004)). In abalone, the full ELH complement remains to be characterised. An alpha-CDCP-like peptide with 94% sequence homology to *L. stagnalis* ELH was identified in the gonad, digestive gland, and cerebral and pleura-pedal ganglia of *H. rubra* (Cummins and Hanna 2004), suggesting similar function between these molluscs. This peptide is known to be involved in egg laying behaviour in *L. stagnalis* in the same way the alpha-BCP peptide acts in *A. californica* (Arch and Smock 1977). Further to this, abalone egg-laying hormones (aELH) have been identified in black lip *Haliotis rubra,* greenlip *Haliotis laevgiata,* and tropical *Haliotis asinina* (Wang and Hanna 1998; Nuurai *et al.* 2010; Botwright *et al.* 2019). Synthetic aELH has been reported to induce spawning in *H. asinina* and *H. laevigata* (Nuurai *et al.* 2010; Botwright *et al.* 2019), however response is likely dependent on exactly which peptide is targeted.

Table 3. Summary of hormone injection spawning trials for Haliotis spp. reported in the literature

Hormone	Species	Number of animals tested	Effect	Reference
Prostaglandin E	*H. rufescens*	9 females, 4 males	Three females and two males spawned within 3.5 h, no controls spawned	Morse *et al.* (1977)
	H. australis	4 females, 4 males	No spawning	Kabir *et al.* (2001)
Prostaglandin F	*H. rufescens*	10 females, 4 males	Five females and two males spawned within 3-16 h, no controls spawned	Morse *et al.* (1977)
Gonadtrophin (mammalian)	*H. rufescens*	7 females, 3 males	No spawning	Morse *et al.* (1977)
Thyroxin (mammalian)	*H. rufescens*	7 females, 3 males	No spawning	Morse *et al.* (1977)
Serotonin	*H. australis*	4 females, 4 males	Two females and one male spawned within two days	Kabir *et al.* (2001)
	H. discus hannai	5 males	More males spawned compared to saline control	Kim *et al.* (2018)
Dopamine	*H. australis*	4 females, 4 males	Two females spawned a small number of eggs	Kabir *et al.* (2001)
APGWamide	*H. discus hannai*	10 males (two concentrations)	More males spawned compared to saline control	Kim *et al.* (2018)
C-terminal amidated ELH	*H. laevigata*	5 females, 5 males	4 females and 4 males spawned within 7-20 h, no controls spawned	Botwright *et al.* (2019)

In addition to ELH, APGWamide and 5-HT are also promising peptides for spawning induction. APGWamide from oyster sperm is also reported to act as a pheromone and can trigger female spawning in oysters (Bernay *et al.* 2006). This is consistent with some aquaculture practices of adding sperm to female abalone tanks for spawning induction (Botwright *et al.*

2019). Administration of 5-HT caused contractions on gonadic tissue of male *H. rubra* that resemble those movements that naturally occur during abalone spawning (Panasophonkul *et al.* 2009). Consequently, 5-HT was proposed to participate in molluscan reproduction (Panasophonkul *et al.* 2009) and recently reported to be involved in oocyte maturation and spawning in *H. discus hannai* (Sharker *et al.* 2020). While conserved hormones have been the primary focus of the above studies, a number of other biomolecules are known to fluctuate in concert with the abalone spawning cycle (Wang *et al.* 2016; Mendoza-Porras, Botwright, *et al.* 2017; Mendoza-Porras, Harris, *et al.* 2017). These biomolecules likely serve a range of reproduction-related functions, such as acting as molecular chaperones to prevent peptide unfolding and/or aggregation, providing protection against stress, and participation in gamete production (Wang *et al.* 2016; Mendoza-Porras, Botwright, *et al.* 2017; Mendoza-Porras, Harris, *et al.* 2017). In *H. asinina*, the serine Kazal-type protease inhibitor Has-KPI was found to be abundant in females on the day of spawning, however injection trials demonstrated that it is unlikely to be a direct spawning inducer (Wang *et al.* 2016). In the same study recombinant Has-KPI was able to form stable aggregates with the molluscan neuropeptide LRDFVamide, indicating that the protease may influence reproduction by modulation of neuropeptides (Wang *et al.* 2016). Non-neuropeptide biomolecules may therefore represent another possible means for spawning induction and should be explored in more depth.

Reproductive disruption strategies

Controlled disruption strategies

Producing triploid abalone is one way to prevent gonad development or sterilise the abalone, as triploidy both significantly decreases gametes and results in low fertilization rates (Ruiz-Verdugo *et al.* 2000; Nell 2002). In particular, triploidy is considered a means to prevent unnecessary reproduction of farmed abalone (Kim, Kim, *et al.* 2019). When animals are sterilised, they divert their energy to growth. Dunstan *et al.* (2007) showed that the growth rate of *H. laevigata* triploids is greater than diploids during sexual maturation, as diploids transfer energy and nutrition to gametophyte generation, while in triploid abalone more energy is available for somatic cell growth (Ihssen *et al.* 1990). Triploids have also been reported to have higher growth rates in other mollusc species, including the blue mussel (*M. edulis*) (Brake *et al.* 2004), the catarina scallop (*Argopecten ventricosus*) (Ruiz-Verdugo *et al.* 2000), and the Indian oyster (*Crassostrea madrasensis = Magallana bilineata*) (Mallia *et al.* 2006). Techniques to induce triploidy include chemical, thermal and hydrostatic pressure. Triploid induction is performed at either meiosis I or II through retention of either the first or second polar, respectively. In *H. asinina* it has been reported that it is easier to produce triploids during meiosis II than meiosis I (Norris and Preston 2003). Survival rates and abnormalities have also been reported to be lower in meiosis I triploids in *H. midae* (Stepto 1997) and *H. discus hannai* (Zhang *et al.* 1998) suggesting triploid induction during meiosis II would be more beneficial if applied in the commercial setting.

Sexual hybridisation is an alternative method to induce sterility in abalone and has similar advantages to triploidy for improved production traits as a result of heterosis or hybrid vigour (Elliott *et al.* 2000; Lafarga de la Cruz *et al.* 2011). This may include the generation of triploid animals through hybridisation of diploid with tetraploid animals. However as yet only a small percentage of tetraploids (< 4%) have been produced as a result of blocking polar body I using caffeine and heat shock for triploidy induction in *H. discus hannai* (Mao *et al.* 2000). An alternative is interspecific hybridisation which has been shown to occur naturally in 33 different combinations among wild *Haliotis* sp. in North America, Japan and Australia (Lafarga de la Cruz *et al.* 2011). In contrast laboratory hybridisation trials may successfully produce fertile eggs however survival is often low if they do not hybridise naturally in the wild. For example, the interspecific hybrid abalone between *H. rufescens*♀ × *H. fulgens* ♂ reported a high fertilisation rate of 85-95%, however only 1% survived to juvenile (Leighton and Lewis 1982). In contrast, fertile interspecific hybrids generally possess the same diploid number of chromosomes in a similar karyotype pattern as reported for the commercial Australian ‘tiger abalone’*H. rubra*♀ and *H. laevigata*♂ (Hamilton *et al.* 2004; Botwright 2015). Current literature suggests that sterility through polyploidy while currently inefficient may be more amenable to induction of sterility in abalone than interspecific hybridisation.

Uncontrolled endocrine disruption

Given the recent overall loss of marine biodiversity and the role of endocrine disruption (ED) in vertebrates and invertebrates, a focus on more research into links between pollution and reproductive problems in invertebrates is important (Langston 2020). Endocrine Disrupting Chemicals (EDCs) are chemicals that disturb the production, release, transport, metabolism, binding and action or elimination of the natural hormones important for homeostasis and cellular development (Langston 2020). Molluscan hormones include ovulation hormone (CDCH-1, FMRFamide), epidermal growth factor (L-EGF), trypsin inhibitor (LTI) and APGWamide peptides; vertebrate-like testosterone and oestrogen sex-steroid hormones linked to vitellin production and serotonin (Matthiessen 2008). The mechanisms of molluscan ED may include disturbance to APGWamide (neuropeptide) in the neurosecretory centres of ganglia, altered conversion of testosterone to oestrogen from aromatase inhibition or altered retinoid-X receptor functions (Langston 2020).

EDCs include numerous chemicals such as xeno-oestrogens (e.g. 17α-ethinyloestradiol (EE2), androgens, bisphenol A (BPA), phthalates, nonylphenol (NP), octylphenol (OP), pesticides (e.g. DDT), herbicides (e.g. atrazine, glyphosate), pharmaceuticals (e.g. hormonal contraceptives), cosmetics, metals (arsenic, antifoulant organotins, cadmium, lead, mercury, zinc), polychlorinated biphenyls and polybrominated flame retardants (Langston 2020). Endocrine disruption can occur at very low doses but not at higher exposure concentrations (U-shaped dose response curve), therefore it is important to minimise exposure even at minute environmental concentrations (Langston 2020). Endocrine Disrupting Chemicals have been associated with human cancers and fertility problems, and feminisation/vitellogenesis/intersex abnormalities in wildlife (e.g. seabirds, alligators) and aquatic species (Langston 2020). In other words, ED is a potentially pervasive environmental toxicology problem with negative implications at the population and biodiversity level.

For molluscan species, a range of ED examples include imposex (acquistion of male characteristics by female organisms) in dogwhelk (*Nucella lapillus*) and shell thickening in *Crassostrea gigas* from organotin tributyltin (TBT), superfemales, excessive embryos, delayed hatching, increased egg laying but reduced growth in freshwater snails from BPA, octylphenol, EE2; and oocyte malformation, altered gamete release time in freshwater mussels from DDT/DDE and intersex in clams (*Scrobicularia plana*) from OP, EE2, NP and E2 (Langston 2020). Hermaphroditism, skewed sex ratios and transgenerational effects also occur from BPA in bivalve molluscs (Matthiessen 2008). The imposex problem where female dogwhelks developed a penis (masculinisation) and were unable to reproduce, led to population extinction in many TBT contaminated sites (Langston 2020). It is also important to recognise that some gastropod species exhibit imposex or pseudo-imposex in their natural populations including giant ram's horn (*Marisa cornuarietis*), banded murex (*Hexaplex trunculus*), noble volute (*Cymbiola nobilis*), bat volute (*Cymbiola vespertilio*), and diadem volute (*Melo amphora*) (Titley-O'Neal *et al.* 2011). In these species it is the change in incidence of imposex within a population that is an informative measure of ED, thus highlighting the gravity of ED in sensitive aquatic species.

Pollutants in the marine environment can cause problems with abalone recruitment through ED from TBT and triphenyltin (TPhT) used in antifouling paints (Horiguchi *et al.* 2000). The organotins masculinised 90% of female abalone (*Haliotis madaka*) resulting in intersex animals that have spermatogenesis in ovarian tissue (ovotestis) at 31.8 ng TBT/g and 11.7 ng TPhT (Horiguchi *et al.* 2000). The result was suppressed ovarian maturation causing dysynchrony between male and female gonad maturation during a 7-month caged-exposure at a site with higher abalone (hence environmental) organotin concentrations (Horiguchi *et al.* 2000). The Environmental Quality Standard (EQS) of 2 ng/L TBT is set to offer protection of some mollusc species (Langston 2020). *Haliotis diversicolor supertexta* fertilized eggs exposed to BPA showed lower embryo hatchability, developmental malformation and suppression of metamorphosis behaviour at environmentally relevant concentrations of 0.05, 0.2, 2 and 10 µg/L (Zhou *et al.* 2011). The deformities included cloudy embryo, asymmetric splitting, egg membrane damage, irregular bulging mulberry-like cells, turgid vesicles in perivitelline space and developmental retardation (Zhou *et al.* 2011). The potential mechanisms of toxicity included disruption of osmoregulation, oxidative injury and ED via reduced cell cycle progression by down-regulation of *PC1* gene, and up-regulation of *CB* and *CDK1* genes (Zhou *et al.* 2011). *H. diversicolor supertexta* exposed to BPA (100 µg/L) and diallyl phthalate (DAP, 50 µg/L) which are plastic-associated pollutants, had altered protein activities related to detoxification, oxidative stress, hormone regulation, cellular metabolism and innate immunity functions (Zhou *et al.* 2010).

Reproduction is not the only aspect of ED in abalone. *H. diversicolor supertexta* larvae exposed to NPs (1016.22; 11.65 µg/L) and BPA (30.72; 1.02 µg/L) via a benthic diatom feed source substrate as 12-h and 96-h median effects exposure concentrations (EC_{50}) respectively, resulted in failed embryonic metamorphosis possibly through depression of energy and substance metabolism, cell signalling, formation of cytoskeleton and cilium, immune and stress responses (Liu *et al.* 2011). Skeletal malformations and absence of the peristomial shell occurred at 12h trochophore and the 96h metamorphosis stages, leading to embryonic mortality (Liu *et al.* 2011). The protective concentration of 318.68; 0.99 µg NP/L and 13.93; 0.18 µg BPA/L is based on a hazard concentration for 5% of the species at 12-h and 96-h exposure scenarios respectively (Liu *et al.* 2011). Compared to other aquatic animal species, this abalone species is more sensitive to NP and BPA (Liu *et al.* 2011). These physiological and metabolic responses indicate that ED toxicity from BPA and DAP potentially results in stress in the abalone with negative implications for growth, reproduction and immune functions (Zhou *et al.* 2010).

Ecotoxicology risk assessment and mitigation

The sources of pollutants include sewage treatment works, agricultural run-off, pharmaceutical manufacture, paper mill effluents or the general estuarine pollution; with benthic sediments being a repository of EDCs (Langston 2020). One strategy for control is to monitor for and reduce uptake of EDCs through contaminated water, sediment or feeding sources in the abalone breeding/culture systems. Currently, there is a lack of validated ecotoxicity tests for EDC in mollusc species and more research into the hormone functions of mollusc species is needed to better understand how ED occurs in them (Matthiessen 2008). A range of bioassays are useful for quantifying the ecotoxicological and biological risks from EDCs and they include estrogen receptor (ER) competitive ligand binding assays, oestrogen/androgen receptor-based cell lines, ER-CALUX assay and histology for intersex (Langston 2020). More life-cycle/stage exposure tests using sensitive molluscan species and their susceptible life stages to EDCs need to be developed to properly risk assess ED-end points (Matthiessen 2008). In conclusion, pollutant ED has been demonstrated in abalone as being sensitive to their adverse effects for reproduction and health, in the context of similar responses across mollusc and vertebrate species. Much more targeted research is needed to develop tools that can measure the risks and inform on what measures are needed to protect abalone.

Conclusion

To date, synthetic peptide-based maturation and spawning induction has only been applied in a research setting. Whether these technologies will be economically viable and adopted by industry is unknown, and will rely on the efficiencies gained and cross species capability. Likewise, as industries focus on attaining best aquaculture practice certification for environmental, social, food safety and animal health and welfare sustainability, the practices employed to eliminate maturation and spawning in the future must be in line with social and legislative requirements. Regardless, improved methods for maturation and spawning induction are likely to be of significant benefit where breeding programs rely on the spawning of small numbers of high value selectively bred broodstock.

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