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Sustainable Aquaculture Using Temporal RNA Interference in Crustaceans: The Case of the Insulin-like Androgenic Gland Hormone and Prawn Monosex Culture

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ABSTRACT

Due to over-fishing and deterioration of wild catch, the ever-growing crustacean market is increasingly relying on aquaculture, driving the need for better management techniques. Since most cultured crustacean species exhibit dimorphic growth patterns, the culture of monosex populations (either all-male or all-female) is the preferred approach for gaining higher yields, with the ecological benefit of reducing the risk of invasion by the non-reproducing cultured species. As recently exemplified in prawns, silencing a transcript encoding an androgenic gland-specific insulin-like peptide trough RNAi, has enabled significant yield improvement through all-male monosex cultures that are the progeny of sexually reversed genetic males. The procedure is temporal, not using exogenous hormones and non-genetically modifying (non-GMO), thus, marking the first commercialized, RNAi-based, sustainable biotechnology in the entire aquaculture industry. This tool has the potential to revolutionize prawn production, contributing to higher productivity and income for growers.

***Key words*:** RNAi, Gene silencing, Aquaculture, Insulin-like androgenic hormone, Prawn monosex culture, Giant freshwater prawn

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OVER-FISHING AND AQUACULTURE

Since ancient times, fishing from oceans, lakes and rivers has been a major source of food, a provider of employment and other economic benefits for humanity. Ocean productivity seemed particularly unlimited, however, with the increasing technological capacities of fisheries, it was realized that living aquatic resources, although renewable, are not infinite and need to be properly managed.

Fishery statistics suggest that annual global fish catches have plateaued at roughly 90 million metric tons (MMT) per year (FAO, 2002). However, these data present only a partial picture, since there is evidence that many fish species are overfished or heading towards depletion (Hilborn *et al*., 2003) and in the case of some large fishes, commercial fishing has wiped out 90% of the population (Myers and Worm, 2003). The worldwide decline of ocean fishery stocks has created an urge to expand seafood production through fish farming or aquaculture. The latter has become an increasingly important source of food and between 1992 and 2002, global production of farmed finfish and shellfish almost tripled in weight and nearly doubled in value (FAO, 2003). Currently, fish produced through aquaculture activities account for over one quarter of all fish and roughly 50% of crustaceans consumed by humans worldwide (FAO, 2010). As the human population continues to grow and standards of living to increase, our reliance on aquaculture production as an important source of protein and specialty food will, with high probability, also increase. While there is still an enormous potential for increasing production through aquaculture, it has to be sustainable without impinging environmental integrity. Aquaculture productivity could be increased to a large extent through improved farm management technologies such as monosex culture and the use of genetically improved strains/breeds of fish and shellfish, as is the case for crops and terrestrial farmed livestock.

BIMODAL GROWTH PATTERN AND MONOSEX CULTURE

The monosex culture strategy which was first introduced into fish-based aquaculture in the late 1970s, has become increasingly prevalent ever since (Mires, 1977; Tayman and Shelton, 1978; Beardmore *et al.*, 2001; Devlin and Nagahama, 2002; Gomelsky, 2003). Several attempts have been made to apply this aqua-technology to crustacean cultures (Curtis and Jones, 1995; Sagi *et al*., 1997a). It was soon realized that differences between males and females of the same cultured species in terms of growth rate, alimentary needs and behavioral patterns dictate the need to establish management systems specifically tailored to one sex or the

other. One of the inherent advantages of non-breeding monosex culture populations is that energy is diverted from reproduction to growth. Another is the obvious ecological merit derived from culturing non-breeding monosex populations that minimizes the invasion by cultured populations, which have a negative impact on native natural species, thus reducing natural biodiversity. An example of one such species is the freshwater prawn, *Macrobrachium rosenbergii* (de Man), which has been introduced as an aquaculture species into a number of countries.

Advantages of All-male *M. rosenbergii* Culture

Growth rates of crustaceans under aquaculture conditions may be affected by a wide variety of factors, including gender, sexual maturity and age (Hartnoll, 1982; Botsford, 1985; Aiken and Waddy, 1992). A number of crustacean species exhibit bimodal growth patterns in which males grow faster than females or *vice versa* (Hartnoll, 1982). The first attempt to create a monosex culture of *M. rosenbergii* was carried out in a small-scale cage-culture system, leading to an increase in yields compared to a mixed population (Sagi *et al*., 1986). In addition to the higher yields, the prawns of the all-male population reached market size at a faster rate, thus prolonging the fresh product marketing period and enabling the now-vacant pond area to be used for further production (Sagi *et al*., 1986). Subsequent to this small-scale cage experiment, similar results were obtained when a monosex prawn culture was tested under intensive monoculture conditions in earthen ponds (Cohen *et al*., 1988): the all-male stocking gave higher marketable yields, increased average weights, higher calculated income *per* unit area and shorter times to harvest. An all-male culture of *M. rosenbergii* proved to be economically beneficial with a 60% income increase to growers under Indian conditions (Nair *et al*., 2006). Thus, it has become obvious that an efficient biotechnology for the production of monosex prawn populations is required if the monosex culture strategy is to be economically viable, especially in countries where crustaceans constitute an important source of income (Sagi and Aflalo, 2005).

THE CRUSTACEAN ANDROGENIC GLAND

As suggested over six decades ago (Charniaux-Cotton, 1954), and is widely accepted nowadays, sexual differentiation in crustaceans is based on genetically determined predisposition and is mediated by the androgenic gland (AG; Sagi and Cohen, 1990; Sagi *et al*., 1997b). Okumura and Hara, 2004). Charniaux-Cotton (1954) was the first to suggest a pivotal regulatory role for the AG. She showed that bilateral

AG ablation in *Orchestia gammarella* blocked the differentiation of secondary male characteristics and decreased spermatogenesis. Later, Touir (1977) described the effects of the AG on both primary and secondary male characteristics in a number of decapod crustaceans, and Taketomi *et al*. (1990) showed that injection of AG extracts into the crayfish *Procambarus clarkii* accelerated the appearance of male sexual characters. More recently, it was shown that AG implantation into immature females of the crayfish *Cherax quadricarinatus* inhibited vitellogenesis and promoted growth (Manor *et al*., 2004), and AG implantation into females of the mud crab *Scylla paramamosain* resulted with ovarian regression and oocytes degeneration (Cui *et al*., 2005).

The ultrastructure of crustacean AG cells resembles that of a vertebrate protein-producing rather than that of a steroid-producing cell, thus it appears that unlike in vertebrates, it is proteins rather than steroids that are responsible for the control of sexual differentiation (King, 1964). Histological evidence (Awari and Kiran, 1999) and changes in total protein content in specific AG cell types (Sun *et al*., 2000) in *M. rosenbergii* support this notion. The masculinization effect of the AG on both primary and secondary female characteristics has been thoroughly investigated (Legrand *et al*., 1968; Katakura and Hasegawa, 1983; Hasegawa *et al*., 1993) and purification, identification and full cDNA sequencing of the isopod androgenic gland hormone have been performed (Martin *et al*., 1999; Okuno *et al*., 1999). Similarly, insulin-like androgenic gland hormone encoding transcripts were discovered and fully sequenced in all economically important groups of decapod crustaceans (shrimp, crabs, prawns and crayfish) including the freshwater prawn *M. rosenbergii* (Manor *et al*., 2007; Ventura *et al*., 2009; Chung *et al*., 2011; Mareddy *et al*., 2011; Ventura *et al*., 2011; Ventura and Sagi, 2012).

AG SURGICAL MANIPULATIONS

In male crustaceans — unlike male vertebrates — the endocrine and gametogenic functions are clearly separated into two distinct organs, the AG and the testis, respectively (Ginsburger-Vogel and Charniaux-Cotton, 1982; Charniaux-Cotton and Payen, 1988). Thus, sex differentiation can be manipulated through the removal of the AG or intervention with its regulatory activities, without damaging the gonads.

A study on maturing *M. rosenbergii* males that had been andrectomised at the youngest developmental stage resulted in a high degree of feminization (Nagamine *et al*., 1980). Re-implantation of the

AG into these andrectomised prawns reversed the latter effect. Males andrectomised at later developmental stages were either partially feminized or not feminized at all (Nagamine *et al*., 1980). Furthermore, surgical removal of the AG from juvenile *M. rosenbergii* resulted with complete sex reversal, leading to the development of functional females capable of mating and producing progeny (Sagi *et al*., 1997b). Functional sex reversal of *M. rosenbergii* females by implanting an AG into the youngest and smallest identified female prawns has also been reported (Malecha *et al*., 1992). In both cases, progeny was obtained when fertile sex-reversed animals were crossed with normal prawns, and the sex ratio of the offspring supported the homogametic male theory (Katakura, 1989).

The above described fully functional sex reversal was devised for a biotechnological scheme producing an all-male monosex culture of *M. rosenbergii* under aquacultural conditions. A two-phase biotechnology was developed (Aflalo *et al*., 2006), to produce all-male populations through large scale AG ablation and sex reversal of males, creating neo-females (genetic males with female phenotype). At present, this two-phase biotechnology is being implemented in Vietnam and Thailand (Aflalo *et al*., 2006). In India, the implementation has been combined with strain selection of best aquacultural performing Indian *M. rosenbergii* strains aiming at the establishment of sustainable all-male production of genetically improved strains (Aflalo *et al*., 2012).

Fraught with difficulties, however, this biotechnology is hampered by the low success rate of the microsurgery (~1.3%) and by lengthy (up to ten months) and labor intensive progeny testing (Aflalo *et al*., 2006; Rungsin *et al*., 2006). These hurdles led to the exploration of novel technologies to meet market demands. Manipulation at the level of the AG hormone and its encoding gene expression are such routes being explored.

EVOLUTION OF RNAi FROM BASIC RESEARCH TO APPLIED BIOTECHNOLOGIES

RNA interference (RNAi) has become one of the major functional genomic tools and has revolutionized functional assays for newly discovered genes (Dorsett and Tuschl, 2004). In brief, RNAi involves the administration of double-stranded RNA (dsRNA) homologous to the gene of interest for post transcriptional gene silencing. This dsRNA is processed into approximately 21-nucleotide RNAs, known as small interfering RNAs (siRNAs), by the enzyme Dicer. These siRNAs then serve as a sequence specific locator of the target RNA as part of the endonuclease activity of the RNA-induced silencing complex (RISC). The RISC complex targets

homologous RNAs for degradation (Agrawal *et al*., 2003; Qi and Hannon, 2005; Watson *et al*., 2005). Since its discovery, RNAi has rapidly gained importance as a reverse genetics tool to knock-down expression of targeted genes in plants, lower animals and microorganisms and has paved the way for easy production of null mutants. RNAi-based gene silencing mechanism is conserved across the plant and animal kingdoms (Baulcombe, 2004) and holds promise not only for functional genomics but also for agricultural and therapeutic applications.

In *C. elegans*, RNAi was initiated by simple soaking the worms in a dsRNA containing solution or by feeding the worms dsRNA-expressing *Escherichia coli* (Timmons and Fire, 1998). This was found to be a very potent method, requiring only catalytic amounts of dsRNA per cell to silence gene expression. The silencing spread from the gut to the remainder of the body, and also through the germline to several generations. These phenomena of RNAi have also been demonstrated to occur in many other invertebrates and vertebrates.

RNAi technology is proving to be useful to quickly study the functions of a number of genes in a wide variety of organisms and has been adapted with high-throughput screening formats in *C. elegans* (Kamath *et al*., 2003) and *D. melanogaster* (Clemens *et al*., 2000). Given the fact that RNAi is easy to apply, whole-genome screens by RNAi may become a common method of choice in the near future.

Although highly attractive as a therapeutic approach (Tuschl and Borkhardt, 2002; Song *et al*., 2003), several hurdles must be overcome to successfully introduce RNAi-based therapies into the clinic and biotechnology arenas. Some of these include efficient and safe systemic delivery, avoidance of undesirable off-target effects, and the development of methods for assessing systemic bio-distribution and sub-cellular localization. On the other hand, in non-medical biotechnology, particularly in the engineering of food plants, the stable and heritable RNAi phenotype has great advantage. Transgenic RNAi plants are constructed in the same way as other genetically modified plants, whereas the target gene of interest must be transformed using a binary vector incorporated to the host plant genome, which replicates to produce dsRNA or hairpin RNA (hpRNA) for RNAi induction (Filichkin *et al*., 2007). RNAi could serve as a powerful and diverse technique that manipulates gene expression in a time or tissue selective manner, for example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid gossypol, making them unsuitable for human consumption. RNAi has been used to produce cotton stocks with reduced levels of a key enzyme in gossypol production in the seeds, without affecting its production in other parts of the plant (Sunilkumar *et al*.,

2006). Similar efforts have been directed towards the reduction of the cyanogenic natural product linamarin in cassava plants (Siritunga and Sayre, 2003). Although no plant products that use RNAi-based genetic engineering have yet passed the experimental stage, research and development efforts have successfully reduced the levels of allergens in tomato plants (Le *et al*., 2006) and decreased the precursors of likely carcinogens in tobacco plants (Gavilano *et al*., 2006). Other plant traits that have been engineered by RNAi in the laboratory include the production of non-narcotic natural products (Allen *et al*., 2004), resistance to common plant viruses (Zadeh and Foster, 2004), and fortification of plants with dietary anti-oxidants (Niggeweg *et al*., 2004). Previous commercial products, including the tomato and papaya, were originally developed using antisense technology but likely exploited the RNAi pathway (Chiang *et al*., 2001; Sanders and Hiatt, 2005). As noted above, all the RNAi interventions in plants to date induce stable and systemic gene silencing, and thus the resulting plants are GMOs, requiring long-term research to completely understand the factors contributing to off-target silencing and prevent potential environmental effects. As stated below, these obstacles were bypassed in the case of the prawn all-male monosex biotechnology through the introduction of non-inherited, transient RNAi.

Introducing RNAi into Crustacean Aquaculture – the Case of Temporal RNAi All-male Biotechnology

In crustaceans, the lack of tools for genetic manipulation and the limited information regarding their gene content has made it difficult to follow the mechanistic basis for RNAi. Recent investigation has provided some molecular clues into gene silencing mechanisms in crustaceans: firstly, experiments in shrimp suggested that the dsRNA travels, probably *via* the circulation, from the site of injection to distant tissues (Robalino *et al*., 2005; Lugo *et al*., 2006; Tiu and Chen, 2007). Secondly, it is clear that extracellular dsRNA is internalized into shrimp cells *in vivo*, as evidenced by the outcoming specific gene silencing (Robalino *et al*., 2007). Because the RNAi machinery allows gene silencing in a highly sequence-specific manner with little or no risk of undesired off-target effects, injections of viral gene-specific dsRNA/siRNA into shrimp were suggested to be a powerful and attractive tool to inhibit viral replication and/or protect shrimp from viral infections. Indeed, this strategy has proven to be effective (Shekhar and Lu, 2009), however, it requires repetitive injections of the silencing agent. An improved delivery method is suggested through feeding of dsRNA, proven feasible for prevention and for blocking viral pathogenesis *in vivo* (Sarathi *et al*., 2008), paving the path for the development of dsRNA-based treatments applicable at a commercial scale.

In our laboratory, gene silencing through RNAi has gained momentum in recent years showing significant success when used for functional genomics studies of several crustacean genes (Shechter *et al*., 2008; Glazer *et al*., 2010; Rosen *et al*., 2010; Pamuru *et al*., 2012; Sharabi *et al*., 2013). This technology was also used to silence the crustacean sexual differentiation governing gene *Mr-IAG* in the cultured prawn *M. rosenbergii* (Ventura *et al*., 2009). When performed at an early developmental stage in males identified through the use of molecular sex markers (Ventura *et al*., 2011), *Mr-IAG* silencing induced a full and functional sex reversal of males into neo-females. Successfully mated with untreated males, the neo-females produced all-male progeny (Ventura *et al*., 2012). Compared with a normal mixed population, the growth patterns of the all-male and the males of the mixed population exhibited a similar size distribution, with the exception that the all-male population had twice the number of male individuals (unpublished results, 2012). This marked the first field study of a monosex population derived from a single gene silencing-induced sex reversal. Commercialization of the process has begun in China and Vietnam. The technology does not involve the use of chemicals/hormones and does not involve genetic modification of the target organism. Since the intervention is temporal and performed on parent prawns, it is not transmissible to next generations, thus bypass by the regulatory pipeline required for genetically-modified crops (Stein and Rodriguez-Cerezo, 2010). This approach may be of tremendous applied merit in the aquaculture industry. Moreover, when delivery challenges are met, gene silencing could be applied in numerous other aquaculture applications and could also be included in sustainable solutions for the management of invasive and/or pest crustacean species, where the production of non-reproducing populations is needed.

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