Progress on the genetics of reproductive performance in penaeid shrimp

Ana M. Ibarra *, Ilie S. Racotta, Fabiola G. Arcos, Elena Palacios


Abstract

A typical feature of penaeid shrimp larval production is that a small proportion of females with multiple spawns contribute to the production of the majority of nauplii. There is no evidence for deterioration in the condition of females and in offspring quality over consecutive spawning in a single generation. Multiple spawning capacity is genetically determined and can be a target in selection programs. Predictive phenotypic traits for selection of multiple spawners might be an important tool to increase larval production. Some of these traits already tested for their inheritance include latency to first spawn, number of spawns, fecundity, egg size, egg vitellin, egg acylglycerides, and egg proteins content, and body weight in mature females; and oocyte diameter and ovary maturity in subadult females. The present review focuses on the recent developments on the genetics of reproduction in shrimp, and on presenting what is known of some of the candidate genes involved in the multiple spawning capacities in shrimp: vitellogenin and sinus gland hormones (for which a peptide homology and phylogenetic analyses are included) and some of the enzymes involved in the biosynthetic pathway of non-peptide hormones. Finally, we present advances in the use of quantitative trait loci identification and gene expression technologies – microarrays – on the study of reproductive performance in other organisms, technologies that are expected to advance our understanding of shrimp reproduction in the future as denser genetic linkage maps and sufficient EST markers become available.

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1. Introduction

Optimization of the quality and quantity of larval production is of primary concern for the penaeid shrimp industry. Whereas important improvements have been achieved in terms of nutrition, environmental control, and management (for reviews see Harrison, 1990; Browdy, 1992; Wouters et al., 2001; Racotta et al., 2003), no studies address the issue of developing genetic selection programs directed to increase reproductive performance simultaneously to other productive traits. Most genetic improvement programs for shrimp have focused on growth and disease resistance improvement (Argue et al., 2002; Gitterle et al., 2005a,b; Gitterle et al., 2006; Goyard et al., 2002; Hetzel et al., 2000; Perez Rostro and Ibarra, 2003a,b). However, as it will be seen in this review, the potential and need to improve and optimize reproductive traits in shrimp exist.

Reproduction of captive stocks is conditional for successful domestication. The belief that pond-reared shrimp stocks had a lower reproductive performance has
proven to be wrong and will not be further discussed here (for reviews see Browdy, 1998; Racotta et al., 2003; as well as recent works of Peixoto et al., 2003; Coman and Crocos, 2003). Instead, it has been shown that it is common within a captive population that a high proportion of the females are non-spawners, and that a low proportion of the females have a high spawning frequency, and therefore these are the ones contributing progeny to the next generation. This could have consequences in the medium to long term. The multiple spawning capability of shrimp could represent an advantage in a productive context because less females can be used during nauplii production. However, selection for multiple spawners in closed populations without a strict control of pedigrees would almost certainly lead to a fast accumulation of inbreeding and to its consequences at the production level: inbreeding depression. The fast accumulation of inbreeding will result from the use of low effective numbers of parents (spawners) contributing to the next generations, rapidly increasing the relatedness among individuals in captivity, and increasing the probability of mating individuals related by decent. Therefore, the best approach for selection on high reproductive quality would be one that integrates reproductive traits with for example, growth traits, into a breeding program that utilizes multiple trait selection index methodology for overall merit.

We focus in this review first on discussing the phenotypic and physiological characteristics, and the predictor phenotypic traits associated with multiple spawner females. Later, we present what is known of the genetics of reproduction in shrimp, including quantitative genetics studies and genomic information on candidate genes involved in shrimp reproduction. We conclude with a section of future research that is expected to allow for the identification of the genes involved in reproduction as well as in the identification of quantitative trait loci associated with the multiple spawning capacity in shrimp.

2. The advantageous phenotype of multiple spawning capacities in production

The advantages of multiple spawners were recently reviewed by Racotta et al. (2003) but since, further work has been published (Arcos et al., 2003a, 2004, 2005a; Coman and Crocos, 2003; Palacios and Racotta, 2003). The capacity of females to produce multiple spawns, in fact equivalent to a higher spawning frequency, is a trait that is commonly measured as a reproductive indicator (Emmerson, 1980; Aquacop, 1983a; Bray et al., 1990; Palacios et al., 1999a). However, multiple spawning capacity is usually reported per female when tagging is done to follow up individually on females, whereas the spawning frequency is generally reported as the average per tank. The final output, in terms of larvae per physical unit (female or tank) within a certain period of time, will depend directly on the number of larvae per spawn and on the number of spawns per physical unit within this period of time. Although fecundity (number of eggs per spawn), fertilization, and hatching rates (determining the number of nauplii produced) can be variable, the largest variation found between females is in the number of spawns produced (Palacios et al., 1999a). This is illustrated in Table 1, where a substantial

Table 1
Reported multiple spawning capabilities of penaeid shrimp; percentages of non-spawning females and of multiple spawners, maximum number of spawns, and their contribution to offspring production

<table>
<thead>
<tr>
<th>Non-spawning females</th>
<th>Multiple-spawning femalesa</th>
<th>Maximum number of spawns</th>
<th>Contribution to offspring productionb</th>
<th>Species and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>66% (Pond-reared)</td>
<td>1.7% (Pond-reared) (≥3)</td>
<td>5 spawns/70 days</td>
<td>P. monodon (Menasveta et al., 1993)</td>
<td></td>
</tr>
<tr>
<td>22% (Wild)</td>
<td>27% (Wild) (≥4)</td>
<td>4 spawns/60 days</td>
<td>P. monodon (Menasveta et al., 1993)</td>
<td></td>
</tr>
<tr>
<td>61–89%c</td>
<td>3.3% (≥4)</td>
<td>7.5%→42%</td>
<td>P. monodon (Menasveta et al., 1994)</td>
<td></td>
</tr>
<tr>
<td>70–90% (Pond-reared)</td>
<td>10% (≥3/moult cycle)</td>
<td>13 spawns/87 days</td>
<td>P. paulensis (Cavalli et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>14–36% (Wild)c</td>
<td>46% (≥2)</td>
<td>19 spawns /100 days</td>
<td>P. paulensis (Cavalli et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>39%</td>
<td>10% (≥3/moult cycle)</td>
<td>5 spawns/60 days</td>
<td>P. semisulcatus (Browdy and Samocha, 1985a)</td>
<td></td>
</tr>
<tr>
<td>24%</td>
<td>46% (≥2)</td>
<td>11%→39%c</td>
<td>P. stylirostris (Bray et al., 1990)</td>
<td></td>
</tr>
<tr>
<td>34% (Pond-reared)</td>
<td>4% (Pond-reared) (≥10)</td>
<td>9%→32%c (p-r)</td>
<td>P. vannamei (Palacios et al., 1999a)</td>
<td></td>
</tr>
<tr>
<td>20% (Wild)</td>
<td>11% (Wild) (≥10)</td>
<td>11%→39%c (wild)</td>
<td>P. vannamei (Palacios et al., 1999a)</td>
<td></td>
</tr>
<tr>
<td>43%</td>
<td>12% (≥3)</td>
<td>5 spawns/60 days</td>
<td>P. vannamei (Peixoto et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>20–33%c</td>
<td>1.2–14%c (≥4)</td>
<td>5 spawns/47 days</td>
<td>P. vannamei (Coman and Crocos, 2003)</td>
<td></td>
</tr>
<tr>
<td>48%</td>
<td>8% (≥4)</td>
<td>7% spawns/36 days</td>
<td>P. vannamei (Arcos et al., 2003a)</td>
<td></td>
</tr>
<tr>
<td>44%</td>
<td>14% (≥4)</td>
<td>6 spawns/29 days</td>
<td>P. vannamei (Arcos et al., 2004)</td>
<td></td>
</tr>
</tbody>
</table>

\[ \text{a: number of spawns indicated in parenthesis; b: proportion of females→proportion of total egg or nauplii production; c: depends on size; d: depends on age; e: these data were not reported in the article but were calculated from original data, p-r: pond-reared.} \]
proportion of females that never spawn can be observed, together with a low number of females with multiple spawns. Comparisons are difficult because different evaluation periods are considered, but apparently a higher multiple spawning capacity exists for open thelycum species. For instance, on a short term basis (1 to 2 months), *Penaeus vannamei* is capable of spawning six to seven times in 29 to 36 days, whereas studies on closed-thelycum species report a maximum of five spawns in 47 to 70 days (Table 1). On a long-term basis, a maximum production of 14 spawns per female in 176 days was reported for *Penaeus semisulcatus* (Browdy and Samocha, 1985b), a value that can be obtained for *P. vannamei* (Palacios et al., 1999a) and *Penaeus stylirostris* (Bray et al., 1990) in less than 100 days.

In any case, the important point is that among shrimp, only a very small proportion of females with multiple spawns contribute to the production of the majority of nauplii, as shown in Table 1 and as reported by Wyban and Sweeney (1991), who observed that 10% or 26% of females contributed to 50% or 75% of the nauplii production, respectively. In contrast to spawning frequency, which is usually reported as a population variable, the proportion of non-spawning and multiple spawning females, as well as the maximum number of spawns recorded for at least one female gives a better idea on the individual reproductive output capacity. This is of interest because it allows evaluating, although indirectly, the production capacity of the system, which can be considered optimal when minimum values of non-spawning females and maximum values of multiple spawning females are attained. Whereas we know that multiple spawning capacity is genetically determined (Ibarra et al., 2005) it can be modified by size, age, origin (wild vs. pond reared stock), and nutrition. All these factors can be potentially controlled during production, particularly origin and nutrition. Although it is difficult to separate between size and age, it is recognized that smaller and younger females produce less spawns than larger and older females, but this is valid up to certain size/age, after which a decline is observed (Cavalli et al., 1997; Coman and Crocos, 2003). The separation between the effect of size and age is possible if pond-reared shrimp of the same age are analyzed: a significant correlation was observed between number of spawns and body weight (Palacios et al., 1999a), larger females spawned more times (Menasveta et al., 1994; Hoang et al., 2002), and females with multiple spawns had a higher body weight (Arcos et al., 2003a; Palacios and Racotta, 2003). The effect of origin (wild vs. pond-reared) can also be confusing because of a superiority of the wild population that can be at least partially attributed to the larger size attained by wild shrimp (Menasveta et al., 1993; Cavalli et al., 1997; Palacios et al., 1999a) or to nutrition previous to stocking in maturation tanks. However, when wild and pond-reared populations of similar size are compared, similar spawning frequencies (Browdy et al., 1986) and proportion of females with multiple spawning (Menasveta et al., 1994; Peixoto et al., 2003) were observed for both rearing origins. The combined effect of size, age, and origin can be also appreciated by a significant correlation between number of spawns and size only for pond-reared population but not for wild ones (Palacios et al., 1999a) or by lower multiple spawning capacity in larger (older) wild shrimp (Cavalli et al., 1997). In both cases and as stated previously, larger wild shrimp are apparently too old, resulting in a lower physiological capacity for consecutive rematurations and spawns. An optimal diet can also increase the maximum number of spawns obtained, as was shown by Marsden et al. (1997) who observed a maximum of six spawns with an experimental diet in contrast to four spawns with a control diet. Similarly, *Artemia* enrichment with essential lipids, in comparison to other live food (squid) or to non-enriched *Artemia*, improved overall reproductive performance, such as the proportion of females that spawned more than once, and the maximum number of spawns attained (Wouters et al., 1999).

In addition to the advantage of stocking production tanks with multiple spawning females, there is also a clear disadvantage of non-spawning females: they occupy space and consume food but do not contribute to the production. In sub-optimal conditions (e.g. shrimp are too small) non-productive females can account for up to 90% (Table 1: Menasveta et al., 1994; Cavalli et al., 1997). However, even for apparently homogenous populations with highly productive females, an important proportion (more than 40%) of non-spawning females is still found (Table 1: Arcos et al., 2003a, 2004; Peixoto et al., 2003). Thus, as summarized by Racotta et al. (2003), efforts to increase postlarvae production must be directed to establishing predictor traits that would allow hatchery operators for the culling and replacement of non-spawning females (McGovern, 1988; Cavalli et al., 1997). However, because the risk of inbreeding is high if selection is carried on by hatchery operators working with penaeid shrimp in closed cycles without maintaining adequate effective numbers and/or pedigree, the establishment of selective breeding programs to optimally improve the multiple spawning capabilities,
simultaneously to other productive traits as growth, is highly recommended.

3. Physiological characteristics of multiple spawners

The exclusive use of multiple spawners could result in the deterioration of the condition of females and in their offspring quality. Such deterioration, termed “reproductive exhaustion” can be the result of the time spent in production after eyestalk ablation, the order or sequence in which the consecutive spawning occurred per se, or to both. Reproductive exhaustion has been observed in most studies, where the effects of time in production and of consecutive spawnings are not so clearly separated because as shrimp that spend more time in maturation tanks are generally assumed to have had more spawns (Racotta et al., 2003). However, recently Palacios and Racotta (2003) demonstrated that after 1 month of production, 50% of females were spawning for the first or second time, while other females had spawned for their seventh time. Furthermore, when the influence of both variables (the time spent in production vs. the order in which consecutive spawnings occur) were simultaneously analyzed by Arcos et al. (2005a) and their effect accounted for, it was confirmed that larval quality decreased only as a consequence of time in production, but it was not affected over consecutive spawning. Other recent studies analyzing production (fecundity, fertilization, and hatching) or biochemical (levels of triglycerides and vitellin in eggs) variables also indicate that offspring quality is not negatively affected over consecutive spawnings (Arcos et al., 2003a, 2004; Palacios and Racotta, 2003; Peixoto et al., 2004).

Apparently, if broodstock maintenance and particularly diet are adequate, there is a minimal or no decline in offspring quality over consecutive spawns. This is confirmed by nutritional studies, where an optimal diet can counteract a decrease in survival to zoea over consecutive spawnings (Marsden et al., 1997; Wouters et al., 1999). As noted by Vázquez-Boucard et al. (2004), broodstock nutrition can explain why a strong decline of lipids in the hepatopancreas was obtained after three spawns in their study in P. indicus, while no changes were observed after more than ten spawns in P. vannamei (Palacios et al., 2000). The simultaneous variation in the capacities of multiple spawning and in the degree of deterioration over consecutive spawns possibly indicates that they are related: a low capacity might result in a quick reproductive exhaustion, whereas when a high capacity is observed, no deterioration in physiological condition of females and offspring quality exists. Our studies in P. vannamei support this hypothesis: The physiological condition of females was not affected over consecutive spawns in terms of ovary development analyzed by histology (Palacios et al., 1999b) or of accumulation of biochemical reserves in ovaries (Palacios et al., 2000; Arcos et al., 2003a). Furthermore, females with multiple spawning capacities apparently had a lower degree of atresia (Palacios et al., 1999b) and higher or unaffected levels of reserves in hepatopancreas and hemolymph (Palacios et al., 2000; Arcos et al., 2003a). Finally, the transfer of biochemical reserves to eggs was not affected over consecutive spawning, and in some cases higher levels of some reserves were observed in eggs from females that had more spawns (Arcos et al., 2003a, 2004; Palacios and Racotta, 2003). These results point to individual physiological capacities that could be traced as secondary phenotypic traits which can be used as predictive indicators of multiple spawning, and that will be presented next.

4. Phenotypic traits that predict the multiple spawning capacities in shrimp

Secondary phenotypic traits associated with multiple spawning capacity, and with good egg quality, must therefore comply with the following criteria: (1) be non-invasive, so that there is no need to sacrifice the broodstock, keeping manipulation and stress to a minimum; (2) be measurable at the beginning of the reproduction cycle; and, (3) be easy to measure or quantify and be reproducible. Several of those secondary traits have in fact been identified already, at the level of the females or their first spawn.

As previously mentioned, larger females produce more spawns, and fecundity (i.e. number of eggs for spawn) has also been positively correlated to spawners’ size (Emmerson, 1980; Ottogalli et al., 1988; Palacios et al., 1998). Thus, if fertilization and hatching rate are not affected by female size, total number of eggs should be much higher for larger females (for review, see Racotta et al., 2003). In addition, body weight (BW) and length (BL) are related for a given population by the mathematical $BW = aBL^b$ (Emmerson, 1980; Arcos et al., 2003a). Emmerson (1980) first proposed a condition index (CI) as a ratio between body weight and length: $CI = BW / aBL^b$ that should be equal to 1 for the population. However, individual variation exists and this condition index assumes that values higher than one (CI > 1) correspond to a good condition, i.e. a higher body weight per length unit than the population average. In contrast, when CI < 1, a poor condition is assumed.
and corresponds to a lower body weight per length unit than the population average (Emmerson, 1980; Arcos et al., 2003a). This condition index when evaluated in recently ablated females was consistently and significantly higher for multiple spawners when compared to females with only one or few spawns (Arcos et al., 2003a). However, its heritability, and phenotypic and genetic correlation with multiple spawning capacities remains to be investigated.

Another phenotypic trait, latency period (days) to first spawn after ablation, or the number of days elapsed between ablation and the first spawn produced by a female, has been shown to be an indicator for the number of spawns a female will have during the production cycle ahead (Palacios et al., 2000; Palacios and Racotta, 2003; Arcos et al., 2003a, 2004). Thus, a cut-off point of females that have not spawned during the first 20 (Arcos et al., 2003a) or 10 days (Arcos et al., 2004) has been suggested. However, this is an analysis of probability, and as such, some females that could have spawned might be eliminated. Furthermore, this cut-off point should be determined for the particular conditions of each population and production laboratory.

An additional phenotypic trait, as vitellogenin (VTG) titers in hemolymph before ablation, might provide with an a priori evaluation of females before even attempting to reproduce them. The rationale of this is that it is expected that females with a more advanced ovary development (higher titers of VTG) will mature sooner, and thus will have their first spawn earlier, which in turn it is known to indicate a higher capacity of multiple spawning. Whereas VTG in hemolymph before ablation have not been studied for their relationship with days to first spawn, it is known that VTG in hemolymph might be an indicator of maturation capacity in P. vannamei (Arcos et al., 2003b). The use of protein levels analyzed in hemolymph to assess the maturation state of females was first proposed by Aquacop (1983b). In agreement, Palacios et al. (2000) found that multiple spawning females at the end of a production cycle had higher protein levels in hemolymph that non-spawning females or females with one spawn. Later, Arcos et al. (2003b) found that at the beginning of the production cycle, females in more advanced maturation stages after ablation also had higher levels of VTG in hemolymph before eyestalk ablation. This finding agrees with the expected higher levels of VTG in hemolymph known to occur during secondary ovarian maturation (Quackenbush, 1989; Wouters et al., 2001), and might indicate that those females with higher VTG levels before ablation were in a more advanced stage of maturation, with eyestalk ablation accelerating the process.

Female spawns can also be analyzed in relation to their production (fecundity; fertilization, and hatching rates) and biochemical variables (energetic and structural components), reviewed by Racotta et al. (2003). In general, it was found that fecundity and energetic (vitellin and acylglycerides) or structural (proteins) components of spawned eggs are higher in multiple spawners than in females with few spawns (Arcos et al., 2003a; 2004), and thus could be used as additional predictive phenotypic traits of multiple spawning capacity.

5. Genetics of multiple spawning capacities and associated phenotypic traits

Until very recently there was only one report (Wyban and Sweeney, 1991) associated to the study of the genetics of multiple spawning capabilities in Penaeids shrimp. Those authors found that multiple spawners produced a larger number of progeny that will become multiple spawners themselves. In recent studies, applying a quantitative genetics approach, our research group has demonstrated that the number of spawns is an inherited trait (Ibarra et al., 2005), in addition to the defined phenotypic ‘predictor traits’ of multiple spawning capability presented in the previous section, namely vitellin and acylglycerides content in first spawned eggs, as well as female latency to spawn early (the number of days elapsing between spawning and eyestalk ablation) (Arcos et al., 2004), (see Table 2A for estimated heritabilities). Whereas the estimated heritabilities for vitellin and acylglycerides contents in eggs had large standard errors, most probably caused by a low effective number of spawners analyzed (only spawning females, and only their first spawns were used in the heritability estimations), the large heritability estimate especially for vitellin content in eggs (VIT), is indicative of the inheritance of this trait. Furthermore, the heritability for VIT content in first spawned eggs in P. vannamei shrimp points toward the importance of this lipoprotein or its precursor, VTG, in predicting high spawning quality. Whereas the negative phenotypic correlation between VIT content in eggs with days or latency to first spawn is not significant, the genetic correlation between those traits was high and also negative (Table 3; Arcos et al., 2004). This indicates that a genetic association between this lipoprotein concentration in eggs and the time to first spawn exists.

There are no other reports on the literature dealing with the genetics of reproduction or that of the multiple spawning capabilities in penaeid species. However, the existence of genetic variation for the numbers of spawns
(or number of matings) and associated traits as the number of ovarioles per female, fecundity, and age at first mating among other Arthropods species, particularly the model organism *Drosophila*, has been recognized for some time (Hudak and Gromko, 1989; Wayne et al., 1997; Promislow and Bugbee, 2000; Sgro et al., 2000). Among vertebrates as fish, genetic determination of reproductive traits as egg number, egg size, egg volume, and age at spawning, has also been demonstrated (Gall and Gross, 1975, 1978; Gall et al., 1987; Gall and Huang, 1988; Siitonnen and Gall, 1989; Leary et al., 1989; Huang and Gall, 1990; Sylvén and Elvingson, 1992; Su et al., 1997).

All the traits mentioned for identifying penaeid shrimp females with high frequency and high-quality spawns are usually measured at adult ages at the beginning of the reproduction cycle, but the next question is: Is there an early genetic determination of reproductive quality? For this purpose, a study in a full-sib family structured population of female shrimp *P. vannamei* was conducted (Arcos et al., 2005b). Of particular interest are two findings; the first one being the estimated significant large heritabilities for two traits measured in subadults, ovary maturity and oocytes mean diameter (Table 2B). The second one being the finding that subadult families that had the largest oocyte diameters (e.g. less previtellogenic oocytes) were on the average, when adults, the same families with the largest number of spawns, the least number of days to first spawn after ablation, and the largest total fecundity, all being traits directly measuring the multiple spawning capability of shrimp (Table 4). Based on this information, it appears that females with multiple spawning capabilities begin gametogenesis and primary vitellogenesis earlier than other females when at subadult stages. This implies that the multiple spawning capabilities are genetically determined and established from early gonad development, with a potential indicator of this characteristic being the onset of primary vitellogenesis.

However their analyses require sacrificing the organisms and time-consuming histology. Hence the evaluation of other traits at this early age associated with later reproductive performance is necessary in the future. One such trait in sub-adults could be VTG in hemolymph, which might be a closely correlated trait with later reproductive performance, but this waits to be investigated. Furthermore, and as it will be presented at the end of this review, these early reproductive traits should be investigated for their potential determination-association with one or more quantitative trait loci (QTL), which would allow for the implementation of marker assisted selection programs (MAS) in the future.

### 6. Candidate genes of multiple spawning capacity

Ibarra et al. (2005) proposed a threshold model for studying and explaining the observed number of spawns in a cohort of spawning shrimp. The model derived from...
the fact that, as discussed above, it is common to find a high proportion of females that do not produce a single spawn in the first 30 days after ablation, whereas the remaining females produce variable numbers of spawns. In the threshold model, the cause for females showing increasing number of spawns is proposed to be a consequence of an attained additive genetic value, resulting from hormones transcribed and/or synthesized that control reproduction (negative and positive), amounts of VTG transcription and translation in gonad and hepatopancreas, amounts transported from hepatopancreas into the gonad, and amounts of receptors for hormones and VTG uptake into oocytes. Whereas this is a simplified and basically additive model which does not take into direct consideration environmental effects and many other potentially important genes and their interactions, it allows for the dissecting of possible genetic effectors in the present review, specifically about what is known today of the most evident candidate genes expected to be involved in the multiple spawning capacity in shrimp: the vitellogenin gene ($vg$), the sinus gland hormones ($cHH$-family) genes, and some of the genes coding for enzymes involved in the biosynthetic pathway of specific steroid hormones.

7. Vitellogenin gene ($vg$) expression and VTG synthesis

Based on relatively recent studies on vitellogenin gene ($vg$) expression and quantification of mRNA-VTG in hepatopancreas and ovary (Tseng et al., 2001; Avarre et al., 2003; Tsutsui et al., 2005b), it has been unequivocally demonstrated that transcription of the $vg$ gene in penaeid species occurs in both tissues. For adult penaeids, differential tissue expression of the $vg$ gene or synthesis rate is known to occur in response to eyestalk hormones addition or exclusion, maturation stage, and molt stage of the individual females. For example, Quackenbush (1989) established that for $P.\ vannamei$, eyestalk hormones exclusion induced by ablation is followed by a two-fold higher synthesis rate of VTG in the hepatopancreas than in the ovary. Recently, Tsutsui et al. (2005b) evaluated transcription (VTG-mRNA) of the $vg$ gene in hepatopancreas and ovary during normal maturation in intact adult females of $P.\ japonicus$, finding an increase in VTG-mRNA during gonad development (endogenous and early exogenous vitellogenesis) in both tissues. Similarly, Avarre et al. (2003) observed an increase in VTG-mRNA levels in hepatopancreas and ovary from $P.\ semisulcatus$ females at vitellogenic and late vitellogenic stages when compared to previtellogenic stage in postmolt shrimp. All three studies indicate that both tissues are involved in transcription of the $vg$ gene and synthesis of VTG during penaeid shrimp maturation. However, other results indicate some differences that suggest a more complex regulation of $vg$ genes in hepatopancreas and ovary depending principally on the degree or stage of ovary development. For example, during late exogenous vitellogenesis, the relative levels of VTG-mRNA to total RNA decreased in ovary but not in hepatopancreas compared to earlier maturation stages (Tsutsui et al., 2005b). In addition, an important difference for VTG-mRNA expression between both tissues was observed for previtellogenic stages depending on the particular molt stage: when females were in postmolt and their maturation stage was previtellogenic none of the organs transcribed VTG-mRNA, whereas in intermolt the ovary contributed with larger amounts of VTG-mRNA than the hepatopancreas for females with previtellogenic oocytes as the most abundant type (Avarre et al., 2003).

Whereas differential tissue expression of VTG is known to occur in adult penaeids associated to maturation and molt stages as previously seen, the specific time (age/size) in the life cycle in which vitellogenesis first occurs, and the conditions needed for first transcription and translation or synthesis of the $vg$ gene(s) in the life...
cycle of penaeids are not completely understood today. However, three recent studies in subadults provide with new information related to the spatial and temporal expression of the vg gene(s). The first one, by Parnes et al. (2004) in subadult P. vannamei, evaluated the amount of vg transcription (VTG-mRNA) in shrimp grown under abnormal environmental conditions (low salinity and artificial diet), finding that transcription of the vg gene did occur at this early age, but only in the ovary. Translation or amount of VTG synthesized was not evaluated, but only late perinucleolus oocytes (previtellogenic) were seen in the subadults, and maturation into late vitellogenic oocytes did not occur in adult sizes under those environmental conditions. The important conclusions provided by the work of Parnes et al. (2004) is that in penaeid shrimp, as in Drosophila (Bownes et al., 1993) both, ovary spatial and temporal expression of the vg gene, and environmental conditions seem to have an effect on vitellogenesis. The second study, done in P. vannamei but under normal salinity (35 psu; and feeding an artificial diet), provides evidence for the fact that primary vitellogenesis does begins at sizes between 17 and 21 g in this species. Arcos et al. (2005b) evaluated stages of gonad development and ovary maturity in subadult shrimp, finding that, as in the study by Parnes et al. (2004), previtellogenic oocytes were abundant, but primary vitellogenesis was also underway at this early ages, evidenced by the presence of oil globule stage oocytes among some females. The third study, by Tsutsui et al. (2005a) using immature intact, not ablated cultured shrimp of P. japonicus subadults (14–20 g at normal salinity), found that transcription of the vitellogenin gene (VTG-mRNA) in the hepatopancreas occurred at very low levels, whereas it was negligible in the ovary. However, when subadult females were eyestalk ablated, this resulted in an increase in VTG-mRNA in ovary, but it had no effect on VTG-mRNA levels in the hepatopancreas. This appears to indicate that competence of the ovary for transcription of the vg gene at subadult age is negatively controlled by a hormone in the eyestalk (sinus gland) of shrimp, and that this hormone is different from the hormone controlling secondary (and exogenous) vitellogenesis in adults.

In conclusion, the results from the above studies point to differential expression of VTG- mRNA in hepatopancreas and ovary, suggesting that the ovary is the primary site of primary vitellogenesis, but that extravarian VTG production is apparently necessary to complete the full maturation process, or secondary vitellogenesis. They further point toward a significant environmental control of vitellogenesis. Further studies using molecular tools are needed to explore if VTG transcription-translation in one or both tissues has an association to the differential capacities of shrimp to develop ripe gonads in response to eyestalk ablation and thus, to have a higher spawning frequency. To date, this association is supported by the already mentioned correlation between VTG levels in hemolymph and maturation capacities (Arcos et al., 2003b) as well as the already discussed correlation between subadult maturation stage (probably a consequence of higher VTG production at this age) and further reproductive performance in adults (Arcos et al., 2005b).

8. Vitellogenin gene(s) in penaeids

Partial and complete transcribed sequences (cDNA) of the vg gene have been isolated and characterized in several penaeid species through reverse transcription of the expressed (mRNA) vg gene(s), but only one complete genomic sequence has been reported (Table 5). The length of the complete cDNA is consistent among species, between 7898 and 8012 bp, regardless of the site of isolation (ovary or hepatopancreas). Phylogenetic analysis of reported DNA sequences, and peptide sequence homology analyses were done using sequences downloaded from GenBank. Multiple sequence alignments were performed and distances calculated using the algorithm of Higgins and Sharp (1988) method. Phylogenetic analyses was performed using the Neighbor-Joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates (MEGA 3.0 software package) (Kumar et al., 2004). With the multiple sequence alignments of peptide sequences and the calculated distances an UPGMA tree was obtained using DNA-MAN version 4.15 software (Lynnon BioSoft. Copyright©1994–99). (Sneath and Sokal, 1973).

The results of these analyses indicate that one vg gene exists (or has been identified) in both, ovary and hepatopancreas, for all penaeid species but Metapenaeus ensis, a species for which two vg genes do exist (Fig. 1). The vgI gene of M. ensis is known to be expressed in both ovary and hepatopancreas (Tsang et al., 2003), and shares closer homology in peptide sequence (65%) with that in the other penaeids (Fig. 1a), whereas the vg2 gene is only expressed in the hepatopancreas (Tsang et al., 2003; Kung et al., 2004), and only shares 39% homology with the vgI gene. Kung et al. (2004) suggested that the vg2 gene of M. ensis might be a recently evolved protein derived from the vgI gene, because after phylogenetic analysis it was more closely related with the vg genes from crayfish and freshwater prawns than marine penaeid
shrimp. In fact, recent phylogenetic analyses (nuclear and mDNA) of penaeids have found that *M. ensis* is a monophyletic group genetically distant to the other penaeid species (Lefébure et al., 2006; Vazquez-Bader et al., 2004; Voloch et al., 2005) covered in this review.

9. Expression regulation of *vg* genes

Regulation of expression of the *vg* gene(s), for example by binding transcription regulators to specific sequences, could not be studied without the definition of the genomic *vg* gene upstream sequence. The only penaeid for which a complete genomic sequence exists (*vg1*) is *M. ensis*, and despite its genetic distance from other marine shrimp (Fig. 1b), it serves the purpose of illustrating the importance of understanding gene structure. Tsang et al. (2003) found that the 5′ upstream region of the promoter of the *vg1* gene consists of several transcription factor binding sites, including TATA and CAAT sequences, but also a site for binding of Sp1, a transcription regulator that is known to activate transcription in response to extracellular events. Although for most penaeid species the *vg* gene sequence has not been completed to date and therefore the existence of transcription sites for binding and regulating expression are unknown, that knowledge is important because of the already discussed differential expression of VTG-mRNA in the hepatopancreas and ovary of several penaeids at different life-cycle, maturation, and molt stages, indicating regulation of expression by different transcription regulators.

As a result of the observed differential expression of VTG-mRNA associated with maturation and molt stage of females *P. semisulcatus* previously discussed, and because this and other penaeid species can spawn several times within a single molt cycle, Avarre et al. (2003) postulated that there must be one or more hormonal systems involved in the up-regulation of expression of the *vg* gene after a molt, and a differential modulation of expression in each organ during the intermolt period.

10. Hormonal inhibitors and inducers of gametogenesis/vitellogenesis

In as much as the VTG protein titers in hemolymph may function as predictors of multiple spawning capabilities, the role that its transcription and translation rates

<table>
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Fig. 1. Phylogenetic distances (a) and peptide homology (b) trees for the vitellogenin cDNA and gene sequences isolated from ovary and hepatopancreas of different penaeid species, indicating two distinct genes exist only for *Metapenaeus ensis*, but not for any of other studied penaeid species. Obtained from sequences reported to GenBank (see Table 5) for *Penaeus vannamei*, *P. semisulcatus*, *P. japonicus*, *P. monodon*, *P. merguiensis*, and *Metapenaeus ensis*.
have on determining the multiple spawning capabilities of females is probably partial. They might just be a reflection of the effects that other different effectors previously involved in controlling and triggering gametogenesis and vitellogenesis had on establishing the age of vitellogenesis onset. Because multiple spawning capacity correlates largely with a short latency or days to first spawn (Arcos et al., 2003a, 2004), both of which correlate with advanced stages of ovarian development in subadults (Arcos et al., 2005b), multiple spawn capacity must be associated with the mechanism(s) defining the timing and efficiency of turning on the gametogenesis/vitellogenesis processes during the life cycle, as well as the effectiveness of the mechanism involved in yolk production, transport, and uptake by oocytes.

Gonad development in penaeids is thought to be mediated through negative controls exerted by neuropeptidic hormones produced by the X-organ–sinus gland complex (XO–SG) located in the eyestalk of shrimp, positive or inductive effects of hormones synthesized in the mandibular organ (MO), and regulatory effects of neuromodulators from the brain. The effect of extracts from eyestalk sinus gland, and specifically the negative effects exerted by a non-identified gonad-inhibiting hormone (GIH) on penaeid adults oocyte vitellogenesis and VTG synthesis has been described elsewhere (Soyez et al., 1987; Meusy and Payen, 1988; Quackenbush, 1989; Wilder et al., 1994; Yano, 1998). Also under negative control of the X-organ, but by a second neuropeptide hormone only known to exist to date in crabs, the mandibular organ inhibiting hormone (MOIH), is the synthesis of the isoprenoid methyl farnesoate (MF) in the MO (for review, see Webster, 1998).

11. Genes coding for cHH-like hormones from the XO–SG

The existence of neuropeptidic hormones, thought to exert a negative control on reproduction, has been known for some time (for review see De Kleijn and Van Herp, 1995). Originally discovered in decapods crustaceans within the arthropods, they were all grouped within the name ‘crustacean hyperglycemic hormone family’ (cHH-like hormones) because of their structural similarity, amino acid sequence homology and the conservation of six cysteine residues at the same relative positions. One of the difficulties in identifying specific hormones of the sinus gland has been that they are all synthesized in the same region of the XO–SG complex of the eyestalk (Van Herp and Kallen, 1991), and isolation of one in particular is rarely achieved. Within this cHH-like hormones family the so-called ‘crustacean hyperglycemic hormone’ (CHH) are grouped, as well as the hormones controlling molting and reproduction, MIH, GIH, and MOIH. Today, advances in molecular biology, with the cloning and sequence analyses of several of these neuropeptides and their coded genes (cDNA) in different species, have permitted not only to classify the cHH-like family as one integrated by at least two subtypes, the CHH (type I) and the MIH (type II) subtypes (De Kleijn and Van Herp, 1995; Lu et al., 2000; Chan et al., 2003), but also to identify different genes for each subtype. It has been proposed that all cHH-like neuropeptides derive from an ancestral chh gene by duplication and mutation, and that the number of genes present is expected to increase with evolutionary status of the studied species (Lu et al., 2000; Chan et al., 2003).

Complete cDNA or partial gene sequences of cHH-like hormones for penaeid species reported into the GenBank include the following species: P. chinensis, P. vannamei, P. japonicus, Penaeus monodon, and M. ensis (Table 6). Both subtypes, CHH (type I) and MIH (type II) have been reported for most of those species and more than one sequence also exist within each type. Although some of the reported sequences were not identified as belonging to type I or II, phylogenetic and peptide analyses helped in making this classification. The largest number of existing sequences belongs to the CHH or type I hormones (Figs. 2 and 3). Because of the already mentioned large genetic distance recently found to exist between M. ensis and other penaeid species, the former was not included in the phylogenetic and peptide analyses reported here. From these analyses it has become clear that some sequences that were originally named as MIH (P. vannamei) or type II are in fact CHH or type I hormones. Additionally one reported partial cDNA sequence in the GenBank accession AF312976-for Pacific blue shrimp P. stylirostris fitted with other non-penaeid species, and was not included in these analyses either.

The different groups obtained in the peptide (Fig. 2) and the phylogenetic (Fig. 3) homology trees for CHH sequences are interesting. Based on degree of homology (Claverie and Notredame, 2003), and similarly to what was done for a different peptide by Brandon et al. (2002), it suggests the existence of three different subtypes within the MIH or type II hormones. However, these three subtypes were only found for two of the penaeid species, P. vannamei (Pev SGP-1 vs. Pev SGP-2) and P. japonicus (Pej-SGP-IV vs. MIH-B). For type I or CHH type hormones, seven neuropeptidic hormones subtypes are suggested also by homology (Fig. 2). It is
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<td>C</td>
<td>AF294648</td>
<td>AAL33882</td>
<td>GIH</td>
<td>II</td>
<td>Gu et al., 2002</td>
<td></td>
</tr>
</tbody>
</table>

*Isolated from muscle tissue.
Fig. 2. Homology tree for the aminoacid sequences of the crustacean hyperglycemic hormone family neuropeptides (cHH-like) isolated from sinus gland of penaeid species, indicating the two resulting types with roman numbers (I and II), and the different subtypes within types with Arabic numbers; obtained from sequences existing in the GenBank (see Table 6) for Penaeus vannamei, P. japonicus, P. monodon, and P. chinensis. A different subtype was considered when 75% or smaller homologies were seen.
important to clarify that the existence of several forms or cDNA isolates cannot be interpreted as the existence of more than one type I gene, because it is known that alternative splicing of a single gene transcript, as in crabs (Dircksen et al., 2001) and freshwater prawns (Chen et al., 2004), results in different cDNA isolates.
The hormones known to be directly involved in reproduction in some crustaceans, as the GIH in lobsters and the MOIH in crabs, are known to group within the MIH or type II group of crustacean hyperglycemic hormones (Lu et al., 2000; Chan et al., 2003). Among penaeids, no GIH or MOIH has been identified, and studies investigating the function of isolated MIH subtypes have not been found to play a direct role in reproduction. For example, Pej-SGP-IV type II hormone isolated from *P. japonicus*, whose mRNA level does not change significantly during the molt cycle (Ohira et al., 1997b), has been found to inhibit ecdysteroid synthesis in the crayfish *Procambarus clarkii* (Ohira et al., 1999). When tested in isolated ovaries of adult *P. semisulcatus*, this type II hormone had the lowest inhibition effect among other type I hormones as evaluated on total proteins and vitellin syntheses (Khayat et al., 1998). A novel MIH-B type II hormone has been recently isolated in *P. japonicus*, whose cDNA sequence and coded peptide differ from the previous one mentioned (Ohira et al., 2005), but as Pej-SGP-IV, has been found to have no effect on ovarian transcription of the vg gene in subadults of *P. japonicus* (Tsutsui et al., 2005a).

Interestingly, these last authors found that a cHH-type I hormone, Pej-SGP-III, had a larger inhibiting effect on VTG-mRNA transcription in ovary than the effect exerted by either, Pej-SGP-IV or MIH-B (both belonging to the MIH type II hormones).

In summary, even if significant advances were done in the last decade in the molecular biology of the XO–SG hormones of shrimp species, a controversy over whether a type I or type II hormone in penaeids might be the gonad inhibiting hormone is just appearing in the literature. Other alternative approaches are needed to investigate the endocrine control of reproduction, as for example studies focusing on neuromodulators of XO–SG neurosecretions (for reviews see Fingerman et al., 1994; Fingerman, 1997) and on secondary endocrine glands that are controlled by neuropeptides from the XO–SG complex. This will include the Y-organ (YO) producing ecdysteroids and the MO where MF is synthesized.

12. Other hormones involved in reproduction

Ecdysteroids, mainly 20-hydroxy-ecdysone (20-OH-Ec), are principally involved in the control of molting in crustaceans (for reviews, see Chang, 1985; Lachaise et al., 1993; Wilder and Aida, 1995). In addition, 20-OH-Ec is also involved in regulation of reproductive development, and specifically a role on reinitiating meiosis in previtellogenic oocytes has been demonstrated (Lanot and Cledon, 1989). In addition, it is known that 20-OH-Ec can also stimulate vitellogenesis in *Macrobrachium rosenbergii* (Wilder et al., 1991, 1994) and in the terrestrial isopod, *Oniscus asellus* (Vafopoulou and Steel, 1995). The control of specific genes related to reproduction by 20-OH-Ec is not known for crustaceans, but in *Drosophila* and the mosquito *Aedes aegypti*, transcription of the vg gene(s) is activated by 20-OH-Ec via an interaction with an intracellular receptor (EcR-USP), which is known to act directly upon early expressed genes induced by 20-OH-Ec by binding to specific DNA sequences located in regulatory regions of target genes, known as ecdysteroid-responsive elements (EcREs). Expression of late expressed genes is then turned on by those early expressed genes first induced by 20-OH-Ec (Martin et al., 2001). Whereas in shrimp 20-OH-Ec has not been found to have a specific role in adult vitellogenesis (Mak et al., 2005), the effects of this hormone on transcription of vitellogenin gene has not been studied in a site and age specific form, as for example, in hepatopancreas and ovary at the onset of vitellogenesis (subadults) and during specific maturation stages in adults, particularly during primary vitellogenesis.

A second steroid hormone, methyl-farnesoate (MF), discovered by Laufer et al. (1987) in crustaceans, is considered the analog of the juvenile hormone in other arthropods, and is known to be an inducer of secondary vitellogenesis (for review see Laufer et al., 1993). Accordingly, Mak et al. (2005) found that MF is a regulator of transcription/expression of the vitellogenin gene in hepatopancreas of adult *Charybdis feriatus* crabs during secondary vitellogenesis, although the effects varied among different reproductive stages among the adults.

In contrast to peptide hormones genes, whose products act as the direct effectors on the control of reproduction, the study of other hormones, requiring a complex biosynthetic pathway for their synthesis, relays on studying the molecular biology of the enzymes involved in that biosynthetic pathway. For example, the final step in the biosynthetic pathway of MF involves the methylation of farnesoic acid catalyzed by the enzyme farnesoic acid *O*-methyltransferase (FAMeT). Whereas in penaeid shrimp little is known about this enzyme regulation, in the crab *Cancer pagurus*, the activity of this enzyme is known to be inhibited by MOIH (Weinwright et al., 1998, 1999; cited by Gunawardene et al., 2001). Among penaeids, recent work on the cloning and characterization of the FAMeT-mRNA has found that a differential expression of this enzyme occurs in...
juvenile male and females shrimp, and that the FAMeT gene is not expressed in the eyestalk of juvenile females, but its expression was consistently high on maturing females, when gonadosomatic indices (GSI) increased from 1% to 8% (Gunawardene et al., 2001). This finding appear to support a mechanism for the effect of MF on penaeid reproduction that is similar to the one known to exist in crabs, and the one proposed by Soller et al. (1999) for juvenile hormone in Drosophila melanogaster. In both of these species the role of the juvenile hormone or its analog MF is significant on development of vitellogenic oocytes, including secondary vitellogenesis and oocyte growth. Furthermore, these findings might be related to the differential expression of vg gene in hepatopancreas and ovaries in relation to maturation stage and eyestalk ablation as observed by Tsutsui et al. (2005b) and discussed above.

13. Future trends

Up to this point we have presumed that the multiple spawning capacity in shrimp is the result of a cascade of events, involving many regulatory genes, physiological processes, and environmental conditions. However, one aspect completely unexplored in shrimp reproduction performance is whether a major gene could be involved in determining the significant differences among females for the number of spawns they have. Whereas no definitive evidence exists for a mayor gene to be the cause of the significant differences seen in the multiple spawning capacity of shrimp, there are some results that might point in that direction: the large and significant heritabilities for the traits ‘ovary maturity’ (OM) and ‘oocytes mean diameter’ (OD) in subadults, the significant correlations found for OD and OM with reproductive performance in adults (Arcos et al., 2005b), specifically indicating that the number of spawns at adult sizes was highly and significantly correlated with ovary developmental capacity in subadults, and a distribution of one of those traits (OD) that does not fit normality, and for which no transformation to normality was found (Fig. 4).

In the model organism Drosophila, a quantitative trait loci (QTL) for the number of ovarioles per ovary, a trait related to fecundity, and a second QTL for reproductive success (measured as competition for mating) have been found (Wayne et al., 2001). Further gene expression studies through the use of microarray technology have narrowed down the number of potential genes in the chromosome with the ovariole number QTL to 34 genes. Among these genes are: one for which no previous annotation existed but that might be a target for steroid hormones (GenBank CG17327); a second gene, yellow-f, has significant homology to a family of proteins associated to reaching full reproductive potential in hymenoptera larvae; and a third gene, Suppressor of fused, a member of the hedgehog signaling pathway that affects ovary formation, and that interact genetically with a gene known to affect ovariole number, fused (Wayne and McIntyre, 2002).

QTL loci or major single gene effects associated to reproductive performance have been found not only in Arthropods, but also among vertebrates as fish,
sheep, pigs, and humans, with the most known being the Booroola ‘gene’ in sheep, associated to high prolificacy because of its effects on ovulation rate, and with several genes known today to have strong effects on ovulation rate (see Davis, 2004). For fish species, four QTLs associated to spawning time have been found for rainbow trout (Sakamoto et al., 1999; O’Malley et al., 2003). For shrimp, molecular markers have been in development in the last decade, and genetic linkage maps based on one type of marker, AFLP (amplified fragment length polymorphism) are already out in the literature (Moore et al., 1999; Wilson et al., 2002; Li et al., 2003; Pérez et al., 2004). These developments will soon be followed by linkage maps combined with microsatellites loci, and by the search of QTLs associated to reproduction, growth, and disease resistance.

Up to now, the study of the effectors involved in penaeid shrimp reproduction has been restricted to the study of one effector at a time because of practical and technological difficulties. Whereas single effector’s studies have provided with significant advances as evidenced by the previously discussed sections on vitellogenin particularly, new technologies will provide with a more integrated view of the vitellogenesis process. For example, microarray technology, involving the analysis of differences in gene expression in specific sexes, lines, or in response to particular stimuli, when applied in shrimp, will bring significant advances in the understanding not only of the response genes, but also of effectors involved in controlling and inducing gametogenesis and vitellogenesis in crustaceans, as it has done for Drosophila (Fujii and Amrein, 2002; Cayirlioglu et al., 2003; Parisi et al., 2004).

To reach the same understanding of reproduction as in Drosophila, advances in the development of expressed sequence tags (EST) have to be done in penaeid shrimps. We can cite at least two international groups that are working on the development of ESTs for Penaeus vannamei, one in the USA (contact Paul Gross, Department of Biochemistry, Medical University of South Carolina, http://www.musc.edu/BCMB/facultypages/gr), and the other in Brazil (contact Pedro Galetti, Departamento de Genética e Evolução da Universidade Federal de São Carlos, http://www.shrimp.ufscar.br/datamining/Pedro(1).php). As EST markers become available in microarrays, we expect to achieve a better understanding not only of the physiological processes involved in determining the multiple spawning capacity of penaeid shrimp, but on the specific genes involved in determining its multiple spawning capability.

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