A Sexual Shift Induced by Silencing of a Single Insulin-Like Gene in Crayfish: Ovarian Upregulation and Testicular Degeneration

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Abstract

In sequential hermaphrodites, intersexuality occurs naturally, usually as a transition state during sexual re-differentiation processes. In crustaceans, male sexual differentiation is controlled by the male-specific androgenic gland (AG). An AG-specific insulin-like gene, previously identified in the red-claw crayfish Cherax quadricarinatus (designated Cq-IAG), was found in this study to be the prominent transcript in an AG cDNA subtractive library. In C. quadricarinatus, sexual plasticity is exhibited by intersex individuals in the form of an active male reproductive system and male secondary sex characters, along with a constantly arrested ovary. This intersexuality was exploited to follow changes caused by single gene silencing, accomplished via dsRNA injection. Cq-IAG silencing induced dramatic sex-related alterations, including male feature feminization, a reduction in sperm production, extensive testicular degeneration, expression of the vitellogenin gene, and accumulation of yolk proteins in the developing oocytes. Upon silencing of the gene, AG cells hypertrophied, possibly to compensate for low hormone levels, as reflected in the poor production of the insulin-like hormone (and revealed by immunohistochemistry). These results demonstrate both the functionality of Cq-IAG as an androgenic hormone-encoding gene and the dependence of male gonad viability on the Cq-IAG product. This study is the first to provide evidence that silencing an insulin-like gene in intersex C. quadricarinatus feminizes male-related phenotypes. These findings, moreover, contribute to the understanding of the regulation of sexual shifts, whether naturally occurring in sequential hermaphrodites or abnormally induced by endocrine disruptors found in the environment, and offer insight into an unusual gender-related link to the evolution of insulins.

Introduction

Understanding sexual shifts and differentiation in both vertebrates and invertebrates is of major interest, particularly given our changing environment. In vertebrates, such as fish, the regulation of such processes can be attributed not only to genetics but also to the effects of hormones and/or environmental factors [1,2]. In invertebrates, too, diverse factors are involved in the regulation of sexual differentiation. For example, in arthropods, such as Drosophila melanogaster, gender-biased alternative splicing constitutes an important component of sexual differentiation [3]. Although crustaceans are also considered to be Arthropoda and even believed to be potential ancestors of insects [4], these ancient marine organisms demonstrate regulation of sexual differentiation that is significantly different from that of insects. In Crustacea, the process is governed by the androgenic gland (AG) [5], an endocrine gland restricted to males that secretes male sex hormone(s) [6]. It is believed that female sexual development and secondary characteristics are exhibited only in the absence of the AG, thereby establishing femaleness as a matter of default, negatively regulated by the AG [7]. In crustaceans, AG involvement in inducing masculinity has been thoroughly investigated in several species [8,9]. In the Australian red-claw crayfish, Cherax quadricarinatus, the implantation of AGs into immature females led to the replacement of female characteristics with male traits, as well as to the cessation of vitellogenesis [10–12].

The vast array of observed AG-associated effects has been attributed to an AG-borne hormone. Three orthologs of this putative AG hormone have been isolated from three species of isopods [13,14]. Recently, two uniquely AG-expressed genes were revealed using AG cDNA libraries prepared from the decapods, C. quadricarinatus (Cq-IAG) and the freshwater prawn, Macrobrachium rosenbergii (Mr-IAG) [15,16]. Structurally, both sequences resemble genes of the insulin-like superfamily [15]. Although evidence supporting a role for insulin in sexual differentiation has been previously documented [17], the unique instance of a gender-specific insulin-like gene has thus far been reported exclusively in
crustaceans. Thus, it is possible that insulins may have evolved in the context of regulation of sexual differentiation and not exclusively on the background of metabolism and growth.

AG hormone ineffectiveness, whether induced by AG ablation or resulting naturally from insufficient levels of secreted hormone(s), can account for hermaphroditism, as was suggested previously to explain part of a sexual shift in a protandric amphipod [18]. Generally, intersexuality in sequential hermaphrodites involves a short transition state in which an individual exhibits sex characteristics of both genders. Such sexual intermediacy may also be abnormally induced by chemicals, collectively termed endocrine disruptors. In contrast to the above-described transient intersexuality, *C. quadricarinatus*, a gonochoristic species demonstrating distinct males and females, experiences non-transient intersexuality [19]. This phenomenon has been found in both wild and cultured populations at frequencies ranging from 2–14% [20]. The intersex individuals are genetic females but functional males [21], bearing an active male reproductive system and male secondary sex characters, along with a constantly arrested ovary [22], possibly due to the presence of the AG. It has been shown that upon AG ablation, intersex *C. quadricarinatus* undergo a dramatic morphological and physiological sex shift in which male reproductive organs regress, accompanied by ovarian activation and the onset of vitellogenesis [11,23,24]. Thus, monitoring vitellogenin, an egg yolk precursor protein, in this intersex species could serve as an accurate physiological indicator of sexual shifts [12,24].

Although sexual shifts have been documented in invertebrates [25–28], the physiological mechanisms controlling these events are not fully understood. In these complicated processes, the production of gametes according to the primary gender is stopped, while the production of gametes according to the opposite sex is acquired. Hence, it is probable that in such sexual shifts, the cell population of the gonad is replaced through repopulation with ‘new’ gonia, following the programmed degradation of the ‘old’ gonia via apoptosis. Indeed, apoptosis is responsible for the differentiation of the primordial gonad in response to the tests in zebrafish [29], and several anti-apoptotic factors have already been identified as being crucial for the survival of male rat germ cells [30].

The discovery of the first decapod AG-specific gene in *C. quadricarinatus*, *Cq-IAG* [15], provided a new route for studying crayfish sexuality. Still, the functionality and presumed involvement of *Cq-IAG* in sexual differentiation has yet to be determined. Gene silencing using RNAi offers one strategy to address such questions. This approach has been successful in several crustaceans [16,31]. In the current study, we employ a functional genomics assay that relies on dsRNA silencing of *Cq-IAG* in an intersex model to demonstrate an inducible sex shift. Specifically, male external sex characters were transformed to generate animals that exhibited female maternal care-related traits, along with testicular degeneration and AG hypertrophy (hAG). In parallel, ovarian activation was observed as the result of the onset of vitellogenesis. These findings confirm the pivotal role of *Cq-IAG* in the regulation of sexual differentiation in *C. quadricarinatus*. Significantly, claudication of this role of gene in *C. quadricarinatus* might contribute to understanding of the evolution of the control of processes regulating sexual shifts in protandrous crustaceans and may provide unique insight into the gender-related evolution of members of the insulin superfamily.

**Results**

The isolation of *Cq-IAG*, accomplished via the construction of an AG cDNA library, has been described previously [15]. In the present study, a further screening was conducted. A colony hybridization, which was used to eliminate *Cq-IAG* ESTs from subsequent sequencing, showed that 115 out of 308 colonies were positive for *Cq-IAG*. This result, showing that *Cq-IAG* comprises approximately 25% of the ESTs, is in accordance with the previous screening of the library. Out of the non-*Cq-IAG* ESTs, 177 were randomly picked and sequenced. However, no new genes were identified and thus no further screening was conducted.

A prerequisite for a long *in vivo* assay demonstrating the functionality of *Cq-IAG* was to evaluate the effectiveness of gene knock-down via RNAi. For this purpose, 3 experimental groups were injected with either double stranded RNA (dsRNA) of the targeted gene, exogenous-based dsRNA or carrier only [doubly distilled water (DDW)]. The efficiency of injected *Cq-IAG* dsRNA in reducing *Cq-IAG* expression was revealed by real-time RT-PCR (Fig. 1). Specifically, the relative quantification (RQ) values noted in the experimental group injected with ds-Cq-IAG (0.24*10^5 ± 0.16*10) were at least 30-fold lower (Kruskal-Wallis stat. H (df = 2, N = 18) = 7.450, p = 0.0241) followed by multiple pair-wise comparison, p<0.05) than those of the control group, which was...
injected with DDW (3*10^5 ± 2*10^5). The exogenous Mr-IAG dsRNA (4*10^5 ± 1*10^5) showed a difference that turned out to be non-significant (p = 0.079) from that of the dsCq-IAG group, although a difference of at least one order of magnitude was observed in the raw data. This result showed that this silencing of Cq-IAG expression was specifically induced by Cq-IAG dsRNA injection, in a sequence-dependent manner.

Once the silencing effect of Cq-IAG dsRNA injections had been established, a long-term silencing experiment addressing Cq-IAG functionality was performed, with two experimental young intersex groups- dsRNA-injected and DDW-injected. Silencing of the gene in young intersex animals induced the appearance of maternal care-related secondary sex characteristics. Comparing control intersex crayfish (Fig. 2A) with dsRNA-injected animals revealed increases in the width and length of the endopodite (i.e., the internal branch of the swimming leg) [22] (Fig. 2B). The calculated endopod width index (EWI) of the dsRNA-injected group (1.44 ± 0.130) was similar to that of characterized females, while DDW-injected intersex animals showed a significantly lower EWI (1.01 ± 0.04, Mann Whitney U test, Z = 2.551, p < 0.05), typical of males. Moreover, the male-typical plumose setae, which naturally line the inner side of the endopod of masculine intersex animals (Fig 2D) were clearly transformed in the dsRNA-injected group, which displayed the maternal-care ovigerous simple setae (Fig. 2E) normally exhibited by mature vitellogenic females (Fig. 2F).

After it was shown that the pleopods of Cq-IAG dsRNA-injected intersex animals presented feminized characteristics, the ovaries were dissected and compared for size and coloration. The ovaries obtained from Cq-IAG dsRNA-injected intersex animals were larger than those of control intersex individuals (Fig. 3). Oocytes in the ovaries of Cq-IAG dsRNA-injected intersex animals were yellowish due to the accumulation of yolk (Fig. 3A), as normally would be observed in mature females (Fig. 3B). These oocytes were significantly larger (899 ± 139 μm, Mann Whitney U test, Z = 2.082, p < 0.05) than the whitish oocytes of control animals (Fig. 3C), with an average diameter of 303 ± 77 μm.

Examination of histological sections of the male reproductive system from a dsRNA-injected intersex animal showed an empty sperm duct (Fig. 4A) and degenerating testicular lobules (Fig. 4B). In these animals, there were no lobules at early spermatogenesis stages; the lobules that were present showed arrested spermatogenesis, with only few spermatozoa. In the same animals, these observations were made alongside a mature ovary (Fig. 4C) containing large oocytes, which contained yolk granules (Fig. 4C, D). In contrast, control intersex showed a sperm duct filled with a spermatophore (Fig. 4E) and large active testicular lobules at different stages of spermatogenesis, with highly abundant spermatozoa (Fig. 4F). The ovaries of the control specimens were arrested and contained small primary vitellogenic oocytes (Fig. 4G, H).

Histological examination of the morphology of the AG in the Cq-IAG dsRNA-injected group showed arrest of the male reproductive system and activation of female sexual morphology (Fig. 4), accompanied by hypertrophy of AG cells, with significantly larger nuclei (7.58 ± 0.16 μm, Mann Whitney U test, Z = 4.438, p < 0.001) (Fig. 5D), than in the control group (5.67 ± 0.22 μm) (Fig. 5C). The AG cells in the treatment group also appeared to be larger in their total size. AG hypertrophy in the dsRNA-injected group differed markedly from that induced by ablation of the X-organ sinus-gland complex in the eyestalk [10,32], in which massive production of the Cq-IAG hormone takes place within the AG cells (Fig. 6B, C), possibly due to the

Figure 2. Effects of dsCq-IAG injections on maternal care-related characteristics in intersex crayfish. Pleopods were collected from intersex animals injected with DDW (A) or dsCq-IAG (B) and from mature females (C). Length:width ratios between endopods and exopods of DDW-injected intersex were identical (A), while those of dsRNA-injected animals (B) showed female-like biometrics. Whereas the inner side of the endopod of DDW-injected intersex bore only plumose setae (D), as in males, the inner side of the endopod of dsRNA-injected animals was lined with ovigerous simple setae (E) as is the case of mature females (F). Bottom row (bar = 100 μm) represents an enlargement of the areas defined in squares in the top row (bar = 500 μm).

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Figure 3. Comparison of whole ovaries obtained from dsRNA and DDW-injected intersex crayfish. The whole ovary from a dsRNA-injected intersex animal (A) contained yellowish oocytes, as in a mature female ovary (B), with an average diameter of approximately 900 μm. The ovary of a DDW-injected intersex animal (C) contained whitish oocytes, approximately 300 μm in diameter (bar = 2 mm).

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Figure 4. Effects of dsCq-IAG injection on the reproductive system of intersex individuals. Hematoxylin- and eosin-stained cross-sections used for structure description. Components of the reproductive system of Cq-IAG dsRNA-injected intersex animals showed an empty sperm duct (black arrowhead, A) and inactive testicular lobules (black arrowheads, B), along with an activated ovary containing enlarged yolk-accumulating oocytes (C). A filled sperm duct (E), spermatogenic testis (F) and an arrested ovary (G) were observed in the control intersex animal. Enlarged areas within the ovaries of both groups are shown in the right hand side (D and H). Bar = 500 μm in ovarian sections, 50 μm in sperm duct, testis and ovary, high magnification sections.

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removal of a specific inhibiting agent. On the contrary, AG cells of the hypertrophied Cq-IAG-dsRNA-injected intersex were characterized by low levels of the Cq-IAG hormone (Fig. 6E, F), a possible consequence of the RNAi application.

During vitellogenesis in mature female C. quadricarinatus, the vitellogenin (Cq-Vg) gene is transcribed in the hepatopancreas, and its translated product is mobilized through the hemolymph to the ovaries, where it accumulates. Cq-IAG dsRNA injection induced both Cq-Vg transcription and expression of its encoded yolk protein (Fig. 7). Cq-Vg expression was demonstrated by RT-PCR (Fig. 7A) as a single band when amplified cDNA from the hepatopancreas of dsRNA-injected intersex individuals served as the template. Hepatopancreatic cDNA samples of vitellogenic females and mature males served as positive and negative controls, respectively. The presence of cDNA was ensured by amplifying the C. quadricarinatus 18S rRNA (Cq-18S) housekeeping gene, which served as control for RNA extraction and the RT reaction. The 106-kDa vitellogenin polypeptide, indicative of secondary vitellogenesis, was detected and quantified in the hemolymph by using ELISA (Fig. 7B), showing similar levels in males (7 ± 1 μg/ml) and control intersex animals (9 ± 1 μg/ml) (Fig. 7B). In contrast, hemolymph samples obtained from dsRNA-injected intersex animals showed a significant increase in the level of yolk proteins (8860 ± 5629 μg/ml, Kruskal-Wallis statistic: H (df = 3, N = 22) = 16.137, p = 0.001) followed by multiple pair-wise comparison, p < 0.05) reaching a concentration even higher than that seen in vitellogenic females (1315 ± 308 μg/ml) (Fig. 7B).

Discussion

While previous studies have demonstrated the influence of the AG on a wide array of characteristics related to sexual development and growth of C. quadricarinatus [10,12], only recently a specific AG factor accounting for the male phenotype in this species has been suggested with the identification of Cq-IAG, the first insulin-like AG specific gene to be revealed in decapods [15]. Screening of an AG-derived cDNA library revealed that Cq-IAG comprises about 26% of the ESTs in the library, possibly hinting at the importance of this gene in AG function. Cq-IAG belongs to the insulin-like superfamily of genes, which are generally assigned to processes of metabolism and growth. However, some insulin growth factors are also linked to differentiation and apoptosis [33]. The rare case of Cq-IAG and several other AG-specific insulin-like genes, which are expressed in a gender-specific manner restricted to males, has been thus far recorded exclusively in Crustacea [13–16]. Sequence conservation among crustacean AG-specific insulin-like peptides is relatively low (approximately 16–29%), considering their putative central role as male sex hormones. However, their predicted structures are quite similar and conserved, comprising heterodimeric peptide chains, resulting from the proteolysis of the pro-hormone. Moreover, the various versions of the peptide share the exact same positions of cysteine residues, which govern folding via the formation of disulfide bonds. Our results regarding the induction of femaleness, combined with the demasculinization of male prawn M. rosenbergii
[16], both achieved by the silencing of AG-specific insulin-like genes, indicate that insulin-like genes have evolved not only in the context of metabolism and growth but also in the context of male sex differentiation. However, in substantial difference from the prawn, the present study, addressing an intersex crayfish, suggests that in addition to the induction of maleness, the AG-specific insulin-like factor serves as a gender switcher that controls the maleness/femaleness balance in an intersex organism. This concept gives rise to a hypothesis linking AG-specific insulin-like factors to the regulation of sexual shifts in crustacean sequential hermaphrodites. Although insulin-like genes have been documented in the context of insect sexuality [34], proliferation and growth [35,36], no evidence of a gender-specific insulin-like gene has previously been recorded in this taxonomic group. The fact that such a gender-specific insulin-like gene, playing a pivotal role in crustacean sexual differentiation, does not exist in insects is somewhat puzzling, since crustaceans are considered to be evolutionarily older than insects [4].

An important observation of the present study is the extensive AG hypertrophy seen following Cq-IAG silencing, a procedure that led to low transcription and poor production of the proteinaceous hormone encoded by the Cq-IAG gene. It is thus suggested that the AG-specific insulin-like factor Cq-IAG may, therefore, regulate its own production and secretion by means of feedback inhibition. Thus, the hybridization of Cq-IAG mRNA with the exogenous Cq-IAG dsRNA, which led to the degradation of the corresponding mRNA, permitted only basal levels of protein production and possibly induced compensation, leading to hypertrophy. We cannot rule out the possibility that higher hierarchy levels of regulation (e.g. paracrine and/or endocrine) could possibly also regulate AG activity, thereby inducing the observed hypertrophy, since hypertrophy of a gland in compensation for low levels of secreted product is well documented in cases of endocrine deficiencies [37].

This study rests on the sexual plastic model of intersexuality as the basis for elucidating the role of the AG-specific insulin-like gene in C. quadricarinatus and its involvement in sexual regulation. Cq-IAG silencing in the intersex crayfish clearly revealed the involvement of this gene in maintaining maleness, as reflected in the empty vas deference and arrest of spermatogenesis that resulted from knockdown of Cq-IAG levels. Moreover, the extensive degeneration of the testicular lobules observed indicates that Cq-IAG is essential for male germ cell survival. Such involvement of an insulin-like peptide in testicular germ cell survival has also been documented in rats [30]. Moreover, the effect of Cq-IAG in extending the viability of male germ cells is similar to that seen with the mammalian male sex hormone, testosterone [38], under similar circumstances. Such resemblance implies that Cq-IAG acts in an androgenic way, being required for male gonad differentiation and ongoing spermatogenesis. Importantly, in silenced intersex C. quadricarinatus individuals, male regression occurred simultaneously with ovarian activation and the onset of vitellogenesis, suggesting that a prerequisite for the activation of femaleness is a lack of sufficient levels of the AG factor, which in turn leads to apoptosis and degeneration of the male gonad and sexual shift. The results of this study, which are in accordance with previous ones [16] and which demonstrate the effect of an
insulin-like gene on male sexuality, are based on loss of function experimentation. Moreover, in a gain of function study using a recombinant protein based on the orthologous AG-specific insulin-like gene in females of the isopod *A. vulgare* [39], the induction of masculinity was documented, thus suggesting a direct role for this AG family of genes in governing masculinity. Such a direct effect of an AG-specific insulin-like peptide using recombinant proteins has not been shown in decapods as yet. Due to the lack of such direct evidence, secondary/indirect regulation of masculine differentiation by an AG-specific insulin-like peptide cannot be ruled out.

The sexual shift induced in this study upon manipulation of a single AG gene may provide, for the first time, insight into the mechanisms underlying sexual shifts that occur naturally in many sequential hermaphrodites in the animal kingdom [40], particularly in protandric hermaphrodite crustaceans. While the endocrine mechanism underlying this phenomenon is not yet known, our results suggest that in protandric hermaphrodite crustaceans, a single insulin-like gene is involved in the regulation of the sexual shift. Our findings also allow us to posit that such an insulin-like gene sustains maleness in early life, followed by intrinsic silencing later on, which triggers sex inversion towards femaleness via an intersexual transition state.

The building of a comprehensive picture of the processes of natural sexual shift opens the window for study of abnormal processes of sexuality. Such cases can be induced by man-made environmental pollutants that act as endocrine disruptors [41]. How these substances induce sex abnormalities remains to be elucidated. Thus, addressing mechanisms in naturally occurring sequential hermaphroditism, such as the AG and the secreted insulin-like peptide studied here, could serve to elucidate pathways inducing sex abnormalities in crustaceans, with possible extrapolation to other arthropods. Such insight carries significant applied implications, such as in the possibility of creating non-breeding, all-male crustacean populations [42] via AG-specific insulin-like gene manipulation. Given that males grow larger than females in some commercialized species, the implications of this possibility are clear.

**Materials and Methods**

**AG cDNA library screening**

The previously constructed *C. quadricarinatus* AG cDNA SSH library [15] was further screened. To avoid *Cq-IAG*-repeated sequencing, four plates containing 127 colonies each (each cloned...
with a single EST from the described library) were screened, using a colony hybridization method [43] with a radio-labeled probe. Briefly, a Cq-IAG cDNA probe was synthesized using a random priming labeling mix (Biological Industries). The probe was hydrolyzed to reduce its length to ∼200 b, as described in the DIG Application Manual (Roche Applied Science). The hydrolyzed probe mix was then hybridized to clones that had been fixed to nitrocellulose membranes. The membranes were hybridized overnight, washed, sealed and exposed to BioMax MS Kodak film as described [44]. The films were then developed according to supplier's instructions. Subsequently, 177 colonies that were considered as non-Cq-IAG were picked to liquid LB, grown over night (Qiagen DirectPrep 96 Miniprep) and sequenced as previously described [15].

Animals

Intercalar C. quadricarinatus animals (each weighing 1–5 g and having a single male genital opening and two female genital openings [21,22]) were grown and maintained in circular cages 10 cm in diameter, floating in 40×40×50 cm³ aquariums situated at Ben-Gurion University of the Negev. Young males (15–17 g), mature males and females (40–70 g) of that species were also previously subjected to surgical removal of the X-organ sinus-gland, an endocrine manipulation causing hypertrophy of the AG, as described [10,32]. The water temperature was kept at 25°C±2, and water quality was assured by circulating the system’s total volume through a biofilter. A photoperiod of 14L:10D was given biweekly over a period of 25–30 weeks (2–5 molts from the beginning of the in-vivo assay).

RNA extraction and real-time RT-PCR

RNA was extracted from the AGs of males used in the short-term preliminary in-vivo experiment. Total RNA was isolated with the EZ-RNA Total RNA Isolation Kit, used according to the manufacturer’s instructions (Biological Industries, Beit Haemek, Israel). First-strand cDNA was synthesized by means of reverse transcriptase reaction using the Verso™ cDNA Kit (Thermo Fisher Scientific Inc.) with 1 μg of total RNA. RQ of Cq-IAG transcript levels were obtained using the following primers: IAG F: 5’-GGCCCTCTTCCTCCCTATGTG-3’ and IAG R: 5’-CCAGCCAGACGAGCAGTGTT-3’ with the FastStart Universal Probe Master (Roche Diagnostics GmbH) and Universal ProbeLibrary Probe #144 (Roche). Cq-18S (accession no. AF235966), which was used as a normalizing gene, was also quantified by means of real-time RT-PCR using the primers: qCq-18S_F: 5’- CTGAGAAAAGCGTGACCACATC-3’ and qCq-18S_R: 5’-GCCGGGAGTGGGTAATTT-3’ with the above-mentioned mix and Universal ProbeLibrary Probe #74 (Roche). Reactions were performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster city, CA, USA).

RT-PCR

RNA was extracted from samples of hepatopancreas and AG from mature males, from samples of hepatopancreas from mature females, and from the hepatopancreas and AG of the long-term in-vivo experimental animals, as mentioned above. The RT reaction was performed using M-MLV H minus reverse transcriptase (Promega), with 1 μg of total RNA from each sample according to the manufacturer’s instructions. Cq-Vg (accession no. AF306784) was amplified by means of PCR with REDTaq ReadyMix™ PCR Reaction Mix (Sigma); one cycle at 94°C–3 min; 37 cycles at 94°C–3 s, 58°C–30 s, 72°C–1 min; one cycle at 72°C–10 min) with the forward primer Cq-Vg F: 5’- AACGAGAGCAGTCTTCTTGCTG -3’ and the reverse primer Cq-Vg R: 5’- CAGCTTTGAGCTGTATCCGACACAGG -3’ using cDNA obtained from hepatopancreas. Cq-18S served as the internal control for the RNA extraction and RT reaction. PCR products were separated on 1.3% agarose gel with Tris-acetate EDTA buffer, and bands were documented. AG cDNA was used to amplify Cq-LAG by PCR (one cycle at 94°C–3 min; 35 cycles at 94°C–1 min, 55°C–45 s, 72°C–50 s; one cycle at 72°C–10 min) with the reaction mix described above, using adaptor primers containing a gene-specific sequence flanked by a restriction recognition site and a T7 RNA polymerase sequence. The primers used were the forward T7-NdeI-F IAG: 5’- TATAGACCCTCATTATGGCTGTTAGGTGACCATGGTTG -3’ and the reverse primer T7-XbaI-R IAG: 5’- TATAGACCCTCATTATGGCTGTTAGGTGACCATGGTTG -3’. PCR products were separated as mentioned above. A fragment was excised, purified (Invisorb Spin DNA Extraction, Invitek, Berlin, Germany) and cloned into the pGEM-T easy vector (Promega). Clones containing the insert were isolated, and plasmid DNA was purified using HiYield Plasmid Mini Kit (RBC Bioscience, Taiwan).
Histological preparations

The reproductive systems of the dsRNA-injected and DDW-injected intersex animals were dissected for morphological observation. Sperm ducts, testes, ovaries and AGs were fixed, dehydrated, embedded in paraffin and sectioned as previously described [16]. Sections were stained hematoxylin and eosin and observed under a light microscope.

Immunohistochemistry

For the immunodetection of Cq-IAG, a specific antibody was generated as follows: Briefly, a recombinant Cq-IAG peptide was expressed in E. coli cells, purified using a nickel column (Ni-NTA Superflow Cartridge, Qiagen) and validated by MALDI-TOF. The confirmed recombinant Cq-IAG peptide was injected into a rabbit. The specificity of the antisera was demonstrated by crossreactivity with the recombinant Cq-IAG by means of Western-blot. For the immunohistochemistry, paraffin sections of AGs obtained from endocrinologically induced [10] and dsRNA-injected intersex animals were deparaffinized and rehydrated. Sections were then incubated in citrate buffer (0.5 M, pH 6-30 min in 95°C) for antigen retrieval and washed in phosphate-buffered saline (PBS; 10 mM, pH 7.4). Blocking (2% normal goat serum, 0.1% Triton X 100, 0.05% Tween 20 in PBS) lasted for 1 h at room temperature, followed by incubation with the primary Cq-IAG antibody (1:10,000). Slides were washed in PBS and incubated with a secondary goat anti-rabbit FITC conjugate (1:500 in PBS with 0.2% fish skin gelatin) for 1 h at room temperature. After PBS washes, slides were mounted (DAPI 1:1000 in PBS and 50% glycerol) and imaged using a confocal microscope.

Anatomical, morphological and physiological measurements

The first left pleopod was removed from all intersex individuals at the beginning of the experiment, and the newly regenerated pleopod was removed at the end of the experiment. The widths of the endopod and the exopod were measured to calculate the EWI [22]. The type of setation on the inner side of the endopod was observed by using a light microscope with an objective micrometer. The average diameter of 15 randomly selected oocytes from each ovary was determined under a light microscope with an objective micrometer (x40, ±2.5 μm). Similarly, the average diameter of 15 randomly selected nuclei of cross-sectioned silenced and control AGs were also determined (x1000, ±1 μm). Vitellogenesis levels were quantified by ELISA [23] using an anti-secondary-vitellogenin-specific 106-kDa polypeptide antibody on hemolymph samples collected from dsRNA-injected intersex animals (n = 3), DDW-injected intersex animals (n = 7), mature females (n = 6) and mature males (n = 6).

Statistical analyses

Data are expressed as means ± SEM. Due to the nature of the experiment the analyzed groups were relatively small and not normally distributed. Thus, non-parametric tests had to be used. All statistical analyses of a single parameter compared between 2 groups (e.g. EWI, oocytes diameter and AG nuclei diameter) were analyzed using the Mann Whitney U Test. The three and four groups analyzed in the real time RT-PCR and ELISA experimentation, respectively, were analyzed using the Kruskal Wallis Test followed by the correction of a multiple pair-wise comparison (built-in within the STATISTICA software) as accepted. We took into account that some groups might differ only in a marginally significant manner due to the usage of a multiple non-parametric test, which relies on a ranking principle.

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Author Contributions

Conceived and designed the experiments: OR AS. Performed the experiments: OR RM SW OG AL EDA TV. Analyzed the data: OR RM EDA AS. Wrote the paper: OR AS.

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