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Review

From the discovery of the crustacean androgenic gland to the insulin-like hormone in six decades

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ABSTRACT

Over the past six decades, a unique crustacean endocrine organ, the androgenic gland (AG), has occupied the minds of groups researching *Crustacea* the world over. Unlike male sexual differentiation and maintenance of sexual characteristics in other arthropods, in crustaceans these processes are regulated by the unique male AG. Crustaceans present a particular case in which the gametogenic organ (testis) is clearly separated from the organ regulating sex differentiation (the AG), enabling endocrine manipulations. The AG was first discovered in a decapod species and later investigated in detail not only in decapods but also in amphipods and isopods. The key role of the AG in regulating sex differentiation was subsequently validated in a number of representative species of a wide array of *Malacostraca*. It was in an isopod species that the AG hormone was first discovered. Later, orthologous genes were found in isopods and decapods, with all these genes sharing the key features of the insulin-like superfamily of peptides. This review unfolds the story of the AG and AG-specific insulin-like factors (IAGs) from a historical perspective, highlighting the main achievements in the field and giving a glimpse of future challenges to be addressed.

1. Introduction

Over 60 years ago, a reproductive system accessory endocrine gland was observed for the first time in males of the blue swimming crab *Callinectes sapidus*. This gland was concisely described as a "ductless accessory gland (which) accompanies the posterior vas deferens. . .no function of the gland is determinable from direct observation" (Fig. 1) [14]. At that point, one could only imagine how the story of this so-called 'accessory gland' would unfold. Seven years went by before a possible role for this unique gland was suggested as a result of a pioneering experiment in an amphipod that showed this gland to be a key regulator of male sexual differentiation (Fig. 1) [9]. In 1955, the gland was termed the androgenic gland (AG) [11].

2. Early studies of the androgenic gland function

Until 1953, sexual differentiation studies based on the removal and grafting of gonads failed in crustaceans. However, that year marked a turning point when Charniaux-Cotton successfully removed and grafted gonads of opposite sexes in amphipods [10]. In the following year, she concluded that the AG controls all male sexual characters, both primary and secondary [9].

2.1. Androgenic gland implantation

In her early studies in the amphipod *Orchestia gammarella*, Charniaux-Cotton investigated both external and internal sex characters. She noted that a certain female-specific external sex character did not regenerate in females with AG implants (designated AG^+), as would normally occur after molting following leg amputation in normal or castrated females. She also noted, for the first time, that vitellogenesis,[2] a secondary female sex characteristic, was completely inhibited in AG^+ *O. gammarella* females. Moreover, the gonia in these AG^+ females gave rise to secondary gonia that divided, as in a testis, and formed an all-sperm-cell lineage [8]. In males deprived of their AGs, the testes ceased to function, and spermatogenesis was replaced by oogenesis, suggesting that this is the default function of the crustacean gonia in the absence of AG hormones, regardless of the genomic composition [7].

Some years later, sexual characters were manipulated through removal and grafting of the AG in the terrestrial isopod *Armadillidium vulgare*. As a result of the grafting procedure, the typical brown color of the female body transformed into the grey-black color of the male body, and the gonads of the AG^+ females transformed into testes with fully developed seminal vesicles and vas deferenses. A time frame for successful sex reversal was determined in this

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[2] In *Crustacea*, vitellogenesis is the process in which the major yolk protein precursor vitellogenin (Vg) is produced in the hepatopancreas and mobilized to the ovary, where it is processed to vitellin (Vt), which accumulates in the oocytes. In some species, Vg is also produced in the ovary.

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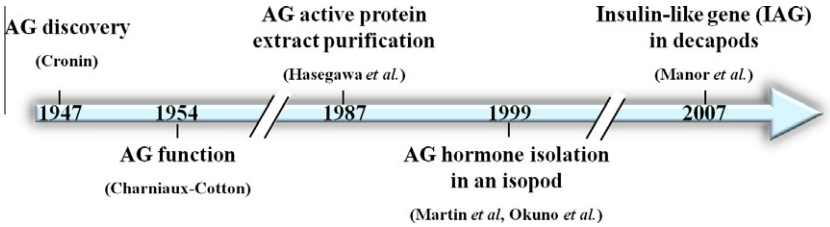

Fig. 1. Key events along the timeline of the research on the androgenic gland. Key events and research groups (in brackets) are indicated along the timeline. The two breaks in the timeline represent the main obstacles – in our opinion – that had to be overcome in AG research: the first obstacle was elucidating the molecular nature of the AG hormone, and the second was the cloning of orthologous genes in decapods.

species – the gonad was found to be bipotent until the females reached a defined life stage [65]. Gonad sex reversal was not documented when AGs were implanted into females with fully differentiated ovaries in *A. vulgare*, in contradiction to the earlier study in amphipods [7]. It should be emphasized that the time-frame-dependent sex reversal in *A. vulgare* is conceptually different from the inhibitory effect of the AG on the fully active female gonad. A more detailed role for inhibition of the female gonad by AG implantation at different life stages was later demonstrated in decapods.

Decapoda, the crustacean order comprising crabs, crayfish, lobsters, prawns and shrimps, has been the focus of AG studies ever since the AG was discovered in a decapod species [14]. In the Australian red-claw crayfish *Cherax quadricarinatus*, AG^+ females demonstrated masculine behavior, as manifested by an increase in aggressive behavior in the presence of normal males [21] and the display of male courtship and false copulations in encounters with intact females [4]. In *C. quadricarinatus*, sexual dimorphism covers a range of characteristics: The females exhibit simple ovigerous setae lining the inner part of the endopod of the pleopods on which the fertilized eggs are carried. In contrast, in males, the pleopod setation is plumose. The female abdomen, on which the fertilized eggs are incubated, is wider than that in males. Both these features, which are distinctive maternal characteristics, were reduced in AG^+ females [36]. The female claw is narrower than the male claw, which is decorated with a red patch on its outer surface, giving the species its common name. Males have been shown to grow faster and reach greater weights. All the above male features were manifested in AG^+ females [26,36]. AG implantation affected several features that are indicators of gonad activity and maturation. In AG^+ females, vitellogenesis cessation was recorded both at the transcript level, by quantitative PCR [36], and at the protein level, by ELISA [58]. The decrease in vitellogenin (Vg) levels led to a sharp decline in oocyte diameter, also reflected by lower gonado-somatic index values [26,36].

In the commercially important giant freshwater prawn *Macrobrachium rosenbergii*, the external sex characters are also prominent. The second pleuron – an indicator of brood chamber size – is significantly wider in sexually mature females than in males. The appendix masculina, a male-specific appendage situated on the second pleopod, buds from the base of the endopod. AG implantation in *M. rosenbergii* females led to a reduction in the width of the second pleuron, the generation of the appendix masculina, and the masculization of the chelipeds, which are preiopods that have differentiated into claws [43]. Ovotestes were observed in females implanted with AGs at relatively early life stages [43]. When AGs were implanted "sufficiently" early, presumably prior to complete ovarian differentiation, a full and functional sex reversal was accomplished, and neo-males were obtained [35].

In the crayfish *Procambarus clarkii*, the first pair of pleopods, which are relatively flexible and slender in females of the species, were modified into gonopod-like, stiff, robust appendages in AG^+ females, as in males [41,68]. Vitellogenesis was inhibited in AG^+ females, as observed by a lack of yolk protein accumulation in the oocytes [68]. In *Eriochier japonicus*, AG^+ female crabs developed male-like appendages [31].

2.2. Androgenic gland ablation

In AG-ablated (designated AG^-) *A. vulgare* males, the overtly external male character – the endopod – which serves as a copulatory organ, ceased to elongate. Vitellogenesis commenced, and Vg titers rose to levels exceeding those of normal females [66]. A complete and fully functional sex reversal of *A. vulgare* was obtained when AG ablation was performed in male larvae. Crossing sex-reversed AG^- males with normal males produced only male offspring, suggesting that males are the homogametic sex in this species [64]. In *O. gammarella* AG^- males, amputated gnathopods regenerated in their undifferentiated, non-masculine form. In these AG^- males, not only did spermatogenesis decrease, but grafted ovaries survived, in contrast to ovaries grafted into normal males (where the AG was present), which rapidly transformed into testes. These observations were attributed solely to the presence or the absence of the AG [7].

In *C. quadricarinatus*, the naturally occurring sexual plasticity – manifested in the form of intersexuality [69] – has been thoroughly documented [59]. Intersex individuals were found to be genetic females [53], although they function as males lacking the ability to undergo any sexual shift. Thus, these intersex animals represent a fascinating case of non-functional hermaphroditism, in which the testis is active and the ovary is permanently arrested. In this species, intersex individuals subjected to AG ablation underwent a dramatic behavioral, morphological, and physiological sex shift. Male-like aggressive and copulatory behaviors were completely feminized in that the AG^- intersex individuals withdrew from fights and did not exhibit mating behavior with receptive females [5]. Moreover, male reproductive organs regressed in parallel with ovarian activation and the onset of vitellogenesis [1,28,58,60].

In the completely gonochoristic species *M. rosenbergii*, on the other hand, AG ablation at different life stages demonstrated a role for the AG in each stage with regard to sexual and morphotypic differentiation. Here, the sexual plasticity is restricted to a narrow window early in the life cycle. Mature *M. rosenbergii* males progress through a succession of three male morphotypes with distinctive external characters [30] and reproduction-related behavior and physiology [54]. Small males transform into orange-clawed intermediate males, which in turn transform into the dominant blue-clawed males. AG ablation of small males and orange-clawed males produced the following findings: While AG^- orange-clawed males transformed into blue-clawed males, AG^- small males did not, suggesting that the AG regulates morphotypic differentiation. The reproductive systems of both AG^- small and orange-clawed males were atrophied, and their somatic growth rates were reduced [57]. AG^- males were de-masculinized

only when AG ablation was performed at early developmental stages—when the male gonopores were visible and before the appendices masculinae had developed. Few molt events following this early AG^{-} in the genetic males, all anatomical female-specific features were developed [42]. Remarkably, several andrectomized juveniles that had undergone a fully functional sex reversal into neo-females (sex-reversed males) and were later mated with normal males produced viable all-male progenies [2,56].

3. The androgenic gland hormone

3.1. Early debates as to the hormone's chemical nature

Twenty years after it was first discovered the AG was found to play a pivotal role in crustacean sex differentiation in all the examined representative *Malacostraca* species. As discussed above, it is well established that the AG is crucial for the development and maintenance of male primary and secondary sex characters (Fig. 1). Although these functions were established relatively fast – providing reliable bioassays in many species – the chemical nature of the AG hormone remained enigmatic for many years. The first clue to its nature came from ultrastructural studies of the AG in the striped shore crab *Pachygrapsus crassipes*. That study suggested the AG hormone was probably a protein or a polypeptide, a supposition based on the observation that AG cells resemble vertebrate protein-producing cells, with an abundant highly developed rough endoplasmic reticulum [29]. This finding was later documented in two other decapod species, *M. rosenbergii* and *P. clarkii* [48,67].

The quest for the identification of AG active substances has drawn considerable attention from the mid-1970s onwards. Researchers were divided in their approaches; some followed the protein link, while others were convinced that the AG active substances were lipoidal in nature. In *M. rosenbergii*, for example, the AG stained positive for lipids, and therefore a steroidogenic nature was suggested for the AG hormone [70]. A lipoidal substance extracted from the AG of the green shore crab *Carcinus maenas* and injected into females of two amphipod species inhibited vitellogenesis. Moreover, in one of the species, male secondary sex characteristics were also produced [6]. This lipoidal substance was later purified and found to be farnesylacetone [17]. At about the same time, a partially purified AG protein extract from *A. vulgare* was found to induce masculinization in injected females [22]. This extract, later understood to be comprised of two protein fractions [19], induced masculinization of young females, including a full and functional transformation of the reproduction system (Fig. 1) [19,23]. Additional studies supporting the protein nature of the AG hormone relied on a loss of activity following proteolysis and amino acid reduction [20,49].

3.2. The proteinaceous AG hormone

The isopod species *A. vulgare* was the central model organism for which efforts to isolate and characterize the AG hormone proved fruitful. The first two protein fractions that were initially found to have androgenic activity in *A. vulgare* (Fig. 1) [19] were analyzed for amino acid composition and found to be in the size range of ~17 kDa. This finding was independently confirmed a few years later [39]. The fact that in both the above studies the two AG fractions were shown to lack cysteine residues is quite puzzling in light of the knowledge obtained on AG hormones since then. However, western blot analysis showed that the above isolated compound was specific to the vas deferens and to the sperm duct but not to the AG [44]. The next major breakthrough in our understanding of the nature of the AG hormone relied on the fractionation method devised by Hasegawa et al. [19], which enabled the full sequence determination of a glycosylated protein [40], in parallel to its characterization and cDNA cloning [51] in *A. vulgare* (Fig. 1). The cloned cDNA was found to be AG specific [51]. The encoded protein was shown to comprise a linear preprohormone, organized according to the insulin-like superfamily of peptides: A signal peptide (which is cleaved off to give rise to the prohormone) at the N′-terminus, is followed by a B chain, then a connecting peptide (C peptide) and finally an A chain. As is common to all insulin-like peptides, the mature hormone has three disulfide bonds, two inter-chain bonds, and one intra-chain bond (within the A chain). In some of the insulin-like peptide subfamilies, the C peptide is cleaved off, thereby converting the prohormone to the heterodimeric active hormone. Several insulin-like peptides also require glycosylation for activity, as is discussed below.

3.3. Post-translational modifications of the hormone

The AG-specific insulin-like peptide from *A. vulgare* was shown to comprise B and A chains interlinked by two disulfide bridges, with another disulfide bridge within each chain. Three active forms were isolated – two glycoforms of interlinked B and A chains and another glycosylated chain with the C peptide still attached. It was suggested that the glycosylated chain was the AG prohormone [40].

Immunoprecipitation of AG extracts with three antibodies raised against different parts of the putative AG hormone (deduced from its cDNA sequence) abrogated AG hormone activity from the extracts when assayed *in vivo* [52], thus supporting the hypothesis that the AG-specific insulin-like peptide is the AG hormone of *A. vulgare* [51]. Lectin affinity chromatography nullified the activity of the AG fraction, thereby giving rise to the hypothesis that the active form of the AG hormone is the glycosylated form [52]. The most significant support for the supposition that the glycosylated form of the AG insulin-like peptide of *A. vulgare* is the active form came from the production of a full-length recombinant protein both in bacteria and moth [50]. The active form was that expressed in the moth after peptidase cleavage of the C peptide. These results provide support for the insulin-like peptidic nature of the mature active hormone, which was found to be active in its glycosylated, C peptide cleaved form. The disulfide bonds found in the active recombinant form were also in keeping with those of the insulin superfamily of peptides [50].

Recently, a semisynthetic *A. vulgare* AG hormone-based glycopeptide was constructed. The non-glycosylated N′-terminus part was produced in bacteria, and the C′-terminus part was synthesized, glycosylated, and then linked to the non-synthetic part [24]. In that study, the glycosylated, semisynthetic mature peptide showed no activity. It was only when the disulfide bridges were "corrected" to the form present in the baculoviral and the refolded bacterial expression systems that it became active. The "correct" disulfide bridges included a linkage between the second and third cysteine residues on the A chain and not the first and third cysteine residues, which is the more thermodynamically stable form found in human insulin. Katayama et al. [24] proposed that this thermodynamically less favorable folding enables the regulation of the mature hormone's biological half-life.

3.4. Isolation of AG-specific insulin-like factors in other species

By means of degenerative primer PCR, two cDNAs encoding highly similar AG specific sequences were identified in two other isopod species – *Porcellio scaber* and *Porcellio dillatatus* [47]. The high sequence similarity of ~90% of the mature hormones is striking, as insulin-like peptides are known to be highly conserved in

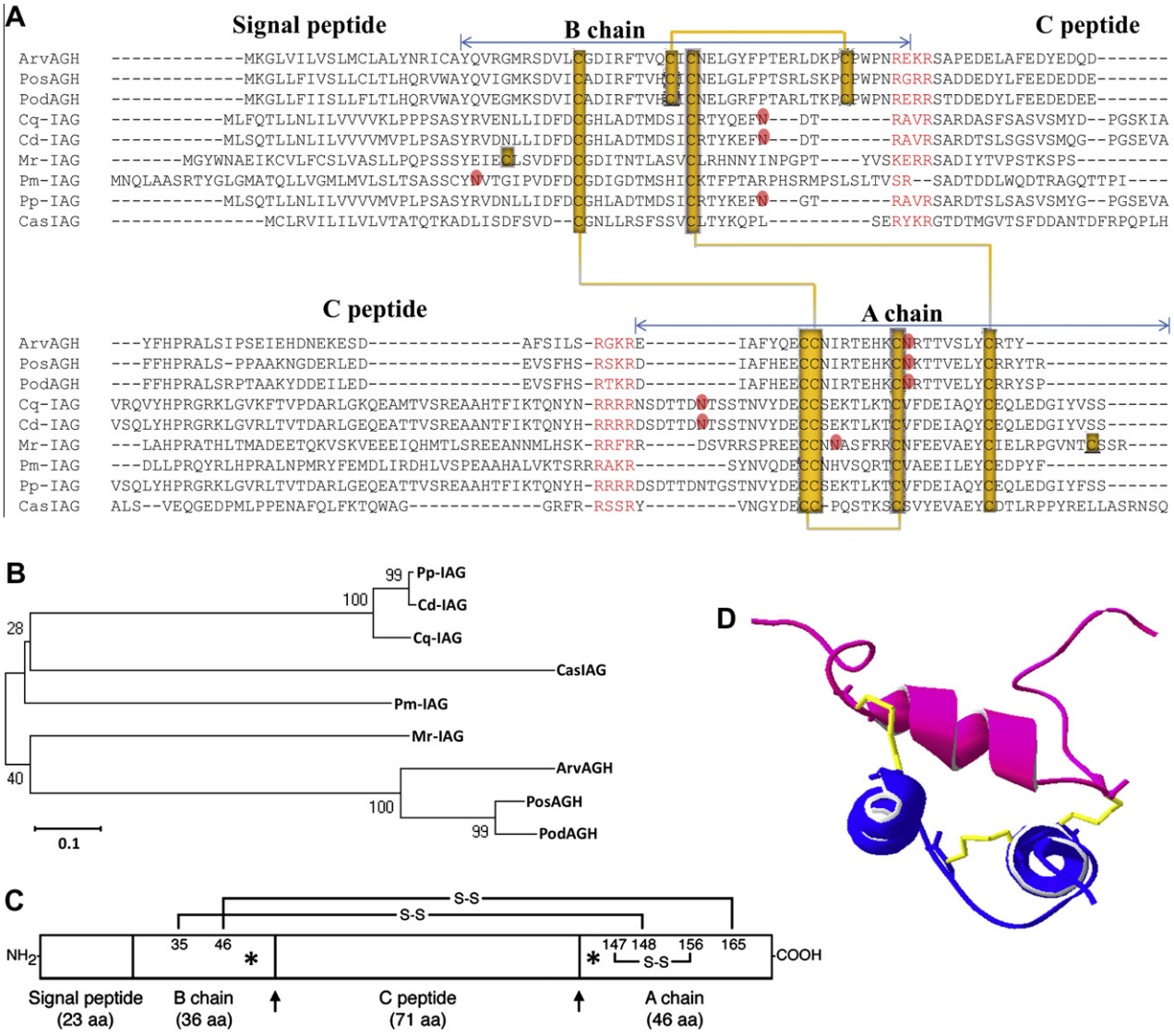

Fig. 2. Insulin-like androgenic gland factors (IAGs): comparison and predicted models of *Cq-IAG*. (A) Multiple sequence alignment of IAGs. All IAGs known to date were aligned by using the CLUSTAL W algorithm, modified by us to fit predicted cleavage sites (RxxR, KxxR or xR; shown in red). The most conserved feature is the backbone consisting of six cysteine residues (highlighted in orange), which gives rise to disulfide bridges (orange lines). Also highlighted in orange are another conserved disulfide bridge that is found specifically in the isopod species and two other cysteine residues in Mr-IAG that might form a third inter-chain disulfide bridge. N-glycosylation sites (predicted in all but ArvAGH) with the sequence of NxS/T are highlighted with red circles. (B) Phylogenetic tree of IAGs. Based on the CLUSTAL W algorithm, all IAGs known to date are represented on a phylogenetic tree (constructed using MEGA4). Isopod IAGs are relatively close to each other but far apart from all other IAGs. Surprisingly, Cd-IAG (from an astacidean crayfish) is closer to Pp-IAG (from a brachyuran crab) than to Cq-IAG (of the same genus – *Cherax*). (C) Linear model of Cq-IAG. Linear model describing the deduced sequence of the components of preproCq-IAG, i.e., the signal peptide, B chain, C peptide and A chain. Arrows indicate predicted Arg C proteinase cleavage sites, at which the C peptide is removed to give rise to the mature hormone consisting of the B and A chains that are interlinked by two disulfide bridges; a third disulfide bridge – an intrachain bridge – is found within the A chain. Asterisks represent two predicted glycosylation sites (NxS/T sequence) at aa 53 in the B chain and aa 137 in the A chain. (D) Three-dimensional model of Cq-IAG. The 3D model of mature Cq-IAG is based on its organizational similarity to bombyxin, an insulin-like peptide from the silk moth *Bombyx mori* (PDB 1BON), for which an NMR structure is available [45]. The 3D model was constructed by EsyPred3D and edited by Swiss-PdbViewer. The peptide backbones of B chain amino acids 24–54 (in purple) and A chain amino acids 142–165 (in blue) are presented in the ribbon diagram. Residues 55–59 (found at the C terminal of the B chain) and residues 131–141 and 166–176 (found at the N and C terminals of A chain, respectively) are not included in the model, because of the differences in length between mature Cq-IAG (82 aa) and bombyxin (48 aa). The side chains of cysteine residues containing disulfide bridges are displayed in yellow. Modified from Manor et al. [37].

both linear and three-dimensional structures but to share low sequence similarity. The three isopod sequences exhibited similarity in all features, including the overall linear sequence, a fourth intra-chain disulfide bridge (in the B chain), which does not occur in all insulin-like peptides, and a predicted glycosylation site in the A chain (Fig. 2A).

Attempts to adopt the molecular shortcut method that proved successful in isopods to isolate AG-specific insulin-like peptides in decapods were unsuccessful for more than 8 years. Then, a new molecular approach that was used to overcome this hindrance took the shape of a subtractive suppression hybridization (SSH) cDNA library [15]. Such an AG cDNA library enabled the first tantalizing discovery of a decapod insulin-like peptide encoding cDNA, which was found in *C. quadricarinatus* and termed *Cq-IAG* (*C. quadricarinatus* insulin-like androgenic gland factor; Fig. 1) [37]. *Cq-IAG* was found to specifically express in the AG *in situ*. It was found to be expressed as early as 8 days after male juveniles were released from the mother. The deduced encoded Cq-IAG had all the characteristics of an insulin-like peptide, in keeping with the structure of the AG specific insulin-like peptides of isopods, with the exception

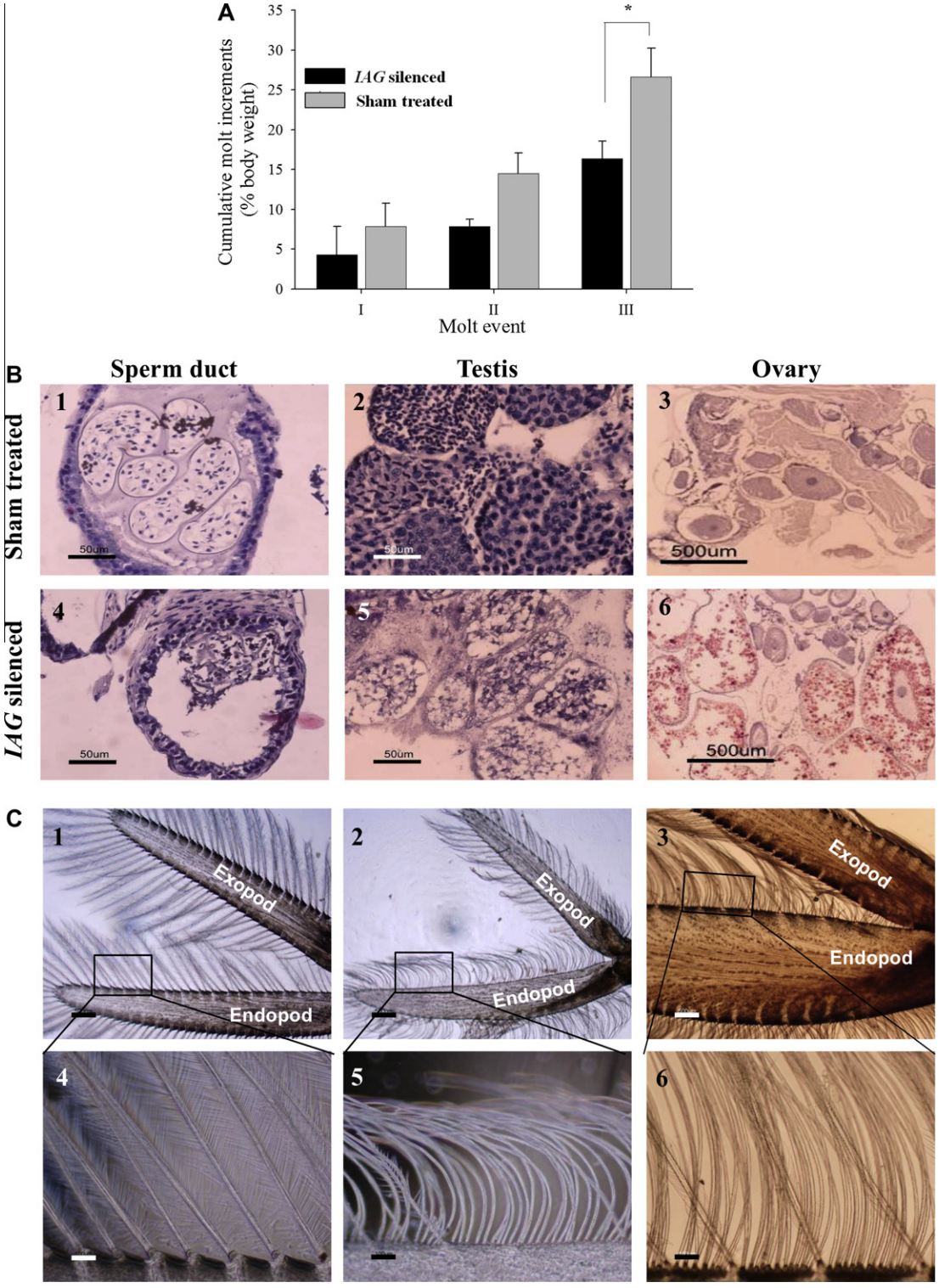

Fig. 3. Effects of *IAG* silencing: growth inhibition, demasculinization and sex shift. (A) Growth inhibition in *M. rosenbergii* males. Cumulative molt increment of sham-treated and *Mr-IAG* silenced groups during three molt events: cumulative molt increment is expressed as 100× weight after molt/weight at start of experiment. Bars represent SEM. Asterisk represents the statistically significant difference observed at the third molt event (paired *t*-test, $p = 0.0224$). Modified from Ventura et al. [71]. (B) Internal sex shift in *Cherax quadricarinatus* intersex. Components of the reproductive system of sham-treated intersex individuals (1–3) appear normal: The sperm duct is filled (1), the testis is spermatogenic (2) and the ovary is arrested (3). In contrast, in *Cq-IAG*-silenced intersex individuals (4–6), the sperm duct is empty (4), testicular lobules are inactive (5) and the ovary (which is permanently arrested in normal intersex individuals) is activated and contains enlarged yolk-accumulating oocytes (6). All sections were stained with hematoxylin and eosin. Modified from Rosen et al. [55]. (C) External sex shift in *Cherax quadricarinatus* intersex. Pleopods were collected from sham-treated (1) and *Cq-IAG*-silenced intersex individuals (2) and from mature females (3). Length:width ratios were identical between endopods and exopods of sham-treated intersex individuals (1), while those of *Cq-IAG*-silenced intersex individuals (2) showed female-like biometrics. Whereas the inner side of the endopod of sham-treated intersex crayfish bore only plumose setae (4), as in males, the inner side of the endopod of *Cq-IAG*-silenced intersex individuals was lined with ovigerous simple setae (5), as is the case of mature females (6). Bottom row (bar = 100 μm) represents an enlargement of the areas defined in squares in the top row (bar = 500 μm). Modified from Rosen et al. [55].

of the additional intra B-chain disulfide bridge. The location of the predicted glycosylation site in the A chain differs from that in the isopods, and there is another predicted glycosylation site in the B chain. The sequence similarity with the isopod peptides is rather low (Fig. 2A and B) [37], which would, in retrospect, explain the previous unsuccessful attempts using the degenerate primers PCR approach. Linear and three-dimensional models of mature Cq-IAG are given (Fig. 2C and D).

The discovery of *Cq-IAG* was only the beginning for more insulin-like encoding genes to be discovered in decapods. The next gene to be discovered was *Mr-IAG*, isolated from *M. rosenbergii*, again by using an SSH cDNA library of the AG [71]. The deduced peptide sequence was not conserved with Cq-IAG and the isopod peptides, but all the insulin-like features were similar, with the exception of a third predicted inter-chain disulfide bridge. *Mr-IAG* was shown to be specifically expressed in the AG. Recently, the sequence of *Pm-IAG* from the commercially valuable tiger shrimp *Penaeus monodon* was revealed through a non-subtractive, full sequence cDNA library of the AG [38]. *Cd-IAG*, which was cloned from the Australian crayfish *Cherax destructor*, shares extremely high sequence identity with *Cq-IAG* (Fig. 2A and B). This similarity was to be expected, because these species are closely related. However, it is quite surprising that in the relatively distant blue swimmer crab, *Portunus pelagicus*, *Pp-IAG* (cloned by means of PCR using a non-degenerative *Cq-IAG*-based primer) showed high sequence identity with IAGs from *Cherax* species (Fig. 2A and B) [63]. This high conservation is yet to be re-examined in light of the evolutionary and developmental distance between the species—crayfish vs. crabs. In Pp-IAG, the predicted N-glycosylation site on the B chain is conserved with that of *Cherax* IAGs. However, there is an additional predicted N-glycosylation site on the A chain of *Cherax* IAGs. Mr-IAG and Pm-IAG, on the other hand, possess single predicted N-glycosylation sites on the A chain and the B chain, respectively (Fig. 2A). The IAG story was recently brought to a symbolic close by the discovery of an AG-specific transcript encoding a deduced insulin-like peptide in *C. sapidus* [12], the species in which the AG was initially discovered over 60 years ago [14].

3.5. IAG functionality in decapods

In terms of functional assays, most of the studies of IAGs conducted in isopods, particularly on the hormone isolated from *A. vulgare*, were based on biochemical approaches. Recently, the utilization of RNA interference (RNAi) has revolutionized the functional assays of newly discovered genes [16]. One such application was the injection of double-stranded RNA (dsRNA) targeting specific genes in different decapods [32,62]. RNAi was used for the functional analysis of the first two decapod *IAG*s identified. In *M. rosenbergii*, *Mr-IAG* was silenced through repeated injections of *Mr-IAG* dsRNA into juvenile males. Upon silencing of this gene in males, regression was observed in growth – in terms of molt intervals and increment (Fig. 3A) – and male characteristics (e.g., regeneration of the appendix masculina and complete cessation of spermatogenesis). The AG-specific expression of *Mr-IAG*, combined with the phenotype observed upon its deprivation, strongly imply that this gene encodes an AG hormone. Moreover, *Cq-IAG* silencing in intersex *C. quadricarinatus* further suggests that the AG-specific insulin-like factor not only regulates male sex differentiation but also plays the role of a gender switcher, balancing male/female components of the intersex crayfish. *Cq-IAG* silencing resulted in the reduction of sperm production and testicular degeneration simultaneously with the onset of vitellogenesis (Fig. 3B) [55]. In the silencing of both *Mr-IAG* and *Cq-IAG*, external sex characters were transformed from male to female, an effect that can be linked – in the case of *C. quadricarinatus* – with acquisition of a maternal care feature (Fig. 3C). The effect of Cq-IAG in extending the viability of male germ cells is similar to that seen for the mammalian male sex hormone, testosterone [46], under similar circumstances. Moreover, the testicular degeneration 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 [25].

4. Concluding remarks and significance

Roughly 60 years ago, Charniaux-Cotton suggested the AG to be the key regulator of reproductive processes such as male sexual differentiation and sexual shifts in *Crustacea*. Evidence supporting this suggestion was subsequently obtained in decapod species by using classical AG^{-} experiments [28,42]. These early classical experiments were succeeded by a new molecular method, which showed AG-specific insulin-like peptide(s) to be the agent(s) bearing the AG activity [55,71].

While IAG transcripts are known in several isopod and decapod species and data are accumulating at the protein level, physiological regulation and genomic data are non-existent. An in-depth understanding of the IAG regulation mechanism will therefore be acquired only after the identification of upstream elements such as the IAG promoter sequence, enhancers, and transcription factors.

In contrast, the endocrine regulation by the AG is fairly well understood. Like other peripheral glands in decapod crustaceans, the AG is thought to be negatively regulated by the X-organ–sinus gland complex (XO–SG) in the eyestalk, which is known to secrete a diverse repertoire of inhibiting neurohormones [61,72]. In *C. quadricarinatus* males, removal of the XO–SG by ablation of the eyestalk resulted in a reduction of the inhibitory effects of the AG [27]. The outcome of such a dramatic violation of the endocrine control over the AG was a significant increase in the size of the gland, accompanied by the production of a differential protein profile (compared with intact males) [27,63,71]. However, to date, there is no documentation of a specific factor isolated from the XO–SG that bears AG-specific inhibiting properties in crustaceans. An additional support for the XO–SG inhibiting effect is given by the recently discovered *CasIAG*, which was found to be negatively regulated by eyestalk-borne components, since eyestalk ablation resulted in its specific elevation [12]. An autocrine feedback inhibition was suggested, as the AG became hypertrophied upon IAG-specific deprivation in *M. rosenbergii* [71] and *C. quadricarinatus* [55]. To further test the latter hypothesis, the receptor binding the IAG should be sought on the membranes of the AG cells. It is predicted that such a receptor, yet to be identified, will also be found in numerous other tissues, since an AG multi-targeting effect has been shown [27,36].

The well-established endocrine regulation of sexual differentiation in crustaceans by the AG was harnessed to induce a sexual shift in gonochoristic species based upon AG ablation in juvenile males. This fully functional sex reversal could be devised for a biotechnological scheme producing *M. rosenbergii* all-male monosex culture under aquacultural conditions [2]. In addition to inducing male sexual differentiation in gonochoristic species, it is possible that IAG plays a role as a gender switcher, balancing male/female states, as has been suggested by observations in intersex crayfish [55]. This concept might link AG-specific insulin-like factors to the regulation of sexual shifts in sequential hermaphrodite crustaceans.

Sexual differentiation and the development of secondary sexual characteristics are controlled by different mechanisms across evolution. In some invertebrate groups, these processes are under the control of both male and female sex hormones [13]. Given the

recent confirmation that insects have no sex hormones [34], the agents responsible for the sexual maturation of this group of animals remain subject to debate. Differentiation of primary and secondary sexual characteristics in insects is thought to be exclusively controlled by the genetic inventory of the individual cell [3]. A recent theory linking the evolutionary paths of *Crustacea* and *Insecta* and defining a new grouping designated *Pancrustacea* [18,33] raises the question as to why there is no mechanism in insects homologous to AG regulation of sexual differentiation in crustaceans.

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