



# The “IAG-Switch” — A Key Controlling Element in Decapod Crustacean Sex Differentiation

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The androgenic gland (AG)—a unique crustacean endocrine organ that secretes factors such as the insulin-like androgenic gland (IAG) hormone—is a key player in crustacean sex differentiation processes. IAG expression induces masculinization, while the absence of the AG or a deficiency in IAG expression results in feminization. Therefore, by virtue of its universal role as a master regulator of crustacean sexual development, the IAG hormone may be regarded as the sexual “IAG-switch.” The switch functions within an endocrine axis governed by neuropeptides secreted from the eyestalks, and interacts downstream with specific insulin receptors at its target organs. In recent years, IAG hormones have been found—and sequenced—in dozens of decapod crustacean species, including crabs, prawns, crayfish and shrimps, bearing different types of reproductive strategies—from gonochorism, through hermaphroditism and intersexuality, to parthenogenesis. The IAG-switch has thus been the focus of efforts to manipulate sex developmental processes in crustaceans. Most sex manipulations were performed using AG ablation or knock-down of the *IAG* gene in males in order to sex reverse them into “neo-females,” or using AG implantation/injecting AG extracts or cells into females to produce “neo-males.” These manipulations have highlighted the striking crustacean sexual plasticity in different species and have permitted the manifestation of either maleness or femaleness without altering the genotype of the animals. Furthermore, these sex manipulations have not only facilitated fundamental studies of crustacean sexual mechanisms, but have also enabled the development of the first IAG-switch-based monosex population biotechnologies, primarily for aquaculture but also for pest control. Here, we review the crustacean IAG-switch, a unique crustacean endocrine mechanism, from the early discoveries of the AG and the IAG hormone to recent IAG-switch-based manipulations. Moreover, we discuss this unique early pancrustacean insulin-based sexual differentiation control mechanism in contrast to the extensively studied mechanisms in vertebrates, which are based on sex steroids.

**Keywords:** androgenic gland, IAG-switch, insulin-like androgenic gland hormone, monosex population, sex determination, sex differentiation, sexual plasticity

## INTRODUCTION

To put the subject of this review into context, we start with a brief history of the discovery of the androgenic gland (AG) in crustaceans. In 1947, in the course of an anatomical/histological study of the male reproductive system in the blue swimming crab *Callinectes sapidus*, an “accessory” endocrine gland was found adjacent to the sperm duct (1). Some years later, this gland was termed the “androgenic gland” in light of its key role in crustacean masculine differentiation, as shown by functional experiments of AG ablation and implantation in the amphipod crustacean *Orchestia gammarella* (2). In later experiments, testis removal in males or implanting females with testicular tissues was found to be ineffective in causing sex reversal, thus suggesting that the vertebrate-like gonadal testosterone is probably not involved in crustacean masculine differentiation (3), and indeed, in 1964, it was first reported that the cells of the AG bore greater similarity to vertebrate protein-producing cells than to steroid-producing cells (4). Thereafter, some functional experiments involving the AG were performed not only by AG grafting but also by injections of AG extracts (5). However, it took a while until a specific AG hormone was first isolated from the terrestrial isopod *Armadillidium vulgare* (6, 7). In 2007, subsequent to the first transcriptomic identification of this hormone in a decapod—the redclaw crayfish *Cherax quadricarinatus*—further validation revealed the hormone structure to be that of an insulin-like peptide (ILP) family member, and the hormone was thus termed the “insulin-like androgenic gland” (IAG) hormone (8). It took about another 10 years before the first report appeared of the successful chemical synthesis of an IAG hormone—that of the giant freshwater prawn *Macrobrachium rosenbergii*, a commercially (9, 10) and environmentally (11) important species (12). Since its first discovery, the IAG hormone has been isolated and characterized in twenty-nine decapod species (Table 1), including prawns, shrimp, crayfish, lobsters and crabs, some of which are highly important for the aquaculture industry worldwide (13), and, as this review will show, the IAG-based sex differentiation mechanism is undoubtedly unique in the Pancrustacea, a diverse taxon that contains all crustaceans and hexapods.

## SEX DETERMINATION AND SEX DIFFERENTIATION IN CRUSTACEANS

In most organisms, sex is determined by chromosomes [i.e., genetic sex determination; GSD (14)] rather than by environmental factors [i.e., environmental sex determination; ESD (15)] (16). The most common GSD systems are the XX/XY and WZ/ZZ systems, in which females are homogametic and males are heterogametic in the former mode of inheritance, and vice versa in the latter (17). With some exceptions (18), most prawn, shrimp and crayfish species bear the WZ/ZZ sex determination system (13, 19–23), while some species of crabs and lobsters bear the XX/XY system (24–27). In the animal kingdom, there are only a few reports of sex-determining genes being associated with the W/Z sex chromosomes. Among

**TABLE 1 |** IAG in decapod crustacean species.

Group	Family	Species	GenBank accession number
Prawn	Palaemonidae	<i>Macrobrachium rosenbergii</i>	FJ409645.1
		<i>Macrobrachium nipponense</i>	JX962354.1
		<i>Macrobrachium vollehovense</i>	KJ524578.1
		<i>Macrobrachium lar</i>	AB579012.1
		<i>Palaemon paucidens</i>	AB588013.1
		<i>Palaemon pacificus</i>	AB588014.1
Lobster	Palinuridae	<i>Sagmariasus verreauxi</i>	KF220491.1
		<i>Jasus edwardsii</i>	KF908794.1
Shrimp	Penaeidae	<i>Litopenaeus vannamei</i>	KX589057.1
		<i>Fenneropenaeus chinensis</i>	JQ388277.1
		<i>Penaeus indicus</i>	MG022137.1
		<i>Litopenaeus occidentalis</i>	KX589058.1
		<i>Litopenaeus stylirostris</i>	KX589059.1
		<i>Marsupenaeus japonicus</i>	AB598415.1
		<i>Penaeus monodon</i>	GU208677.1
		<i>Pandalus platyceros</i>	KX619617.1
	Pandalidae	<i>Pandalus platyceros</i>	KX619617.1
Crab	Varunidae	<i>Hemigrapsus sanguineus</i>	MH580760.1
		<i>Eriocheir sinensis</i>	KU724192.1
	Geryonidae	<i>Chaceon quinquegens</i>	KY497474.1
	Portunidae	<i>Portunus pelagicus</i>	HM459854.1
		<i>Scylla paramamosain</i>	JQ681748.1
		<i>Callinectes sapidus</i>	HM594945.1
		<i>Portunus trituberculatus</i>	MH119940.1
		<i>Carcinus maenas</i>	HM594946.1
	Crayfish	Cambaridae	<i>Procambarus clarkii</i>
<i>Procambarus virginialis</i>			MF405195.1
<i>Procambarus fallax</i>			KX619618.1
Parastacidae		<i>Cherax quadricarinatus</i>	DQ851163.1
		<i>Cherax destructor</i>	EU718788.1

them are the W-chromosome-associated *DM-W* gene, which is vital for ovarian development in the African clawed frog *Xenopus laevis* (28), and the Z-chromosome-linked *DMRT1* gene, whose dosage is assumed to control the sex determination process in the chicken *Gallus gallus domesticus* (29). In contrast to the sparse knowledge on the genes associated with the W/Z chromosomes (especially in crustaceans), the male sex-determining genes that are associated with the Y chromosome and that control masculinization in animals bearing the XX/XY system have been well-characterized. Among these genes, most mammals have the well-known *SRY* gene (30). Other examples include the *DMY/DMrt1bY* gene, which is associated with the formation of the testis in the medaka fish (31), and the recently discovered *iDMY* gene, which is the male sex-determining factor during embryogenesis in the Eastern spiny lobster *Sagmariasus verreauxi* (25). To reveal the genetic content of the sex chromosomes, extensive karyotyping of different decapod crustacean species has been performed and published. However,

none of the available karyotypes can distinguish between the sex and autosomal chromosomes (32–38). Moreover, while genome sequencing using next generation techniques is common and genomes have been published for several decapod species (39–41), none, except that of *M. rosenbergii* (42), is a phased genome in which a certain scaffold could be attributed to a paternal or maternal origin. Therefore, verified sex-determining factors in decapod species, especially those with WZ/ZZ chromosomal content, are yet to be found.

For species in which sex is determined by sex chromosomes (43), the sex differentiation process starts with the expression of genes responsible for promoting masculinization or feminization during early developmental periods. Many such invertebrate genes have been well-studied, including the *mab-3* gene in the nematode *Caenorhabditis elegans* (44), the *transformer-2* (*tra-2*) gene in pancrustaceans (45), and the *doublesex* (*dsx*) gene in the fruit fly *Drosophila melanogaster*, which has alternative spliced variants yielding different sexes (46). Although the information on such sex differentiating genes in crustaceans remains limited, from the few studies that have been conducted, it is known that *dsx* is expressed in the branchiopod *Daphnia magna* (47), and the *dsx* and *mab-3* related transcription factor (*DMRT*) is expressed in the testis of the decapod *Eriocheir sinensis*, the Chinese mitten crab (48). In *M. rosenbergii*, transcriptomic libraries obtained for different developmental stages—from the embryonic stage (49), through larvae and post-larvae, to adults (50, 51)—appear to contain homolog transcripts of the *dsx*, *tra-2*, and *DMRT* genes. Moreover, *IAG* silencing in *M. rosenbergii* resulted in significant decrease in the expression of two *DMRT*s and other sex related genes (52). However, the exact relationship of these genes to the sex differentiation mechanism—if such a relationship does indeed exist—has yet to be found.

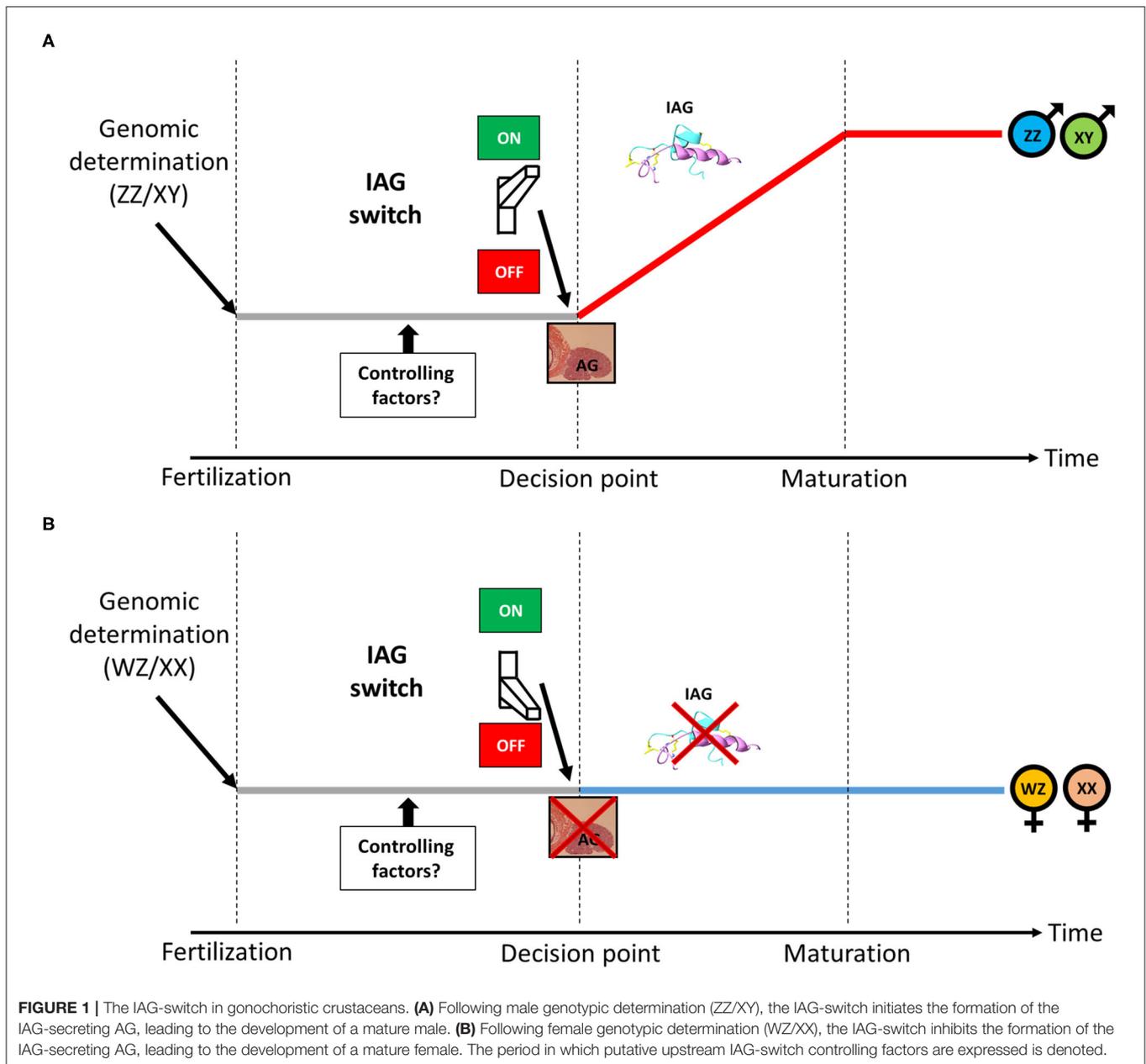
Although in the next section we will describe the universal *IAG* gene as a master switch involved in crustacean sex differentiation, it is noteworthy that sex differentiation mechanisms in crustaceans are not only mediated by genes but also disrupted by external factors. For example, elevated bacterial dosage of *Wolbachia* reduces the functionality of insulin receptors in isopods which results in feminization (53). Additionally, some environmental pollutants serve as endocrine disrupting chemicals (EDCs) suggested to affect sex differentiation and sexual development in crustaceans (54). The latter concept was shown in various crustacean species from different orders. In daphnids, the exposure to DES, a synthetic estrogen, induced the development of secondary sexual characters like larger abdominal process in females of *D. magna*, while longer first antennae were observed in males exposed to the androgen androstenedione (55). Moreover, in *D. pulex*, exposure to methoprene, a juvenile hormone analog, yielded all-female broods, while gravid females exposed to 20-hydroxyecdysone, has resulted in all-male broods (56). In the amphipod *Gammarus pulex*, exposure to the xenoestrogen 17 $\alpha$ -ethynylestradiol increased the females:males sex ratio (57) and in decapods, heavy metals such as cadmium and copper inhibited ovarian growth in the crabs *Uca pugilator* (58) and *Chasmagnathus granulata* (59), while the xenoestrogen 4-nonylphenol reduced testis weight in the crab *Carcinus maenas* (60). A correlation between

endocrine disruptors and crustacean sex differentiation is also exemplified by EDCs discharge in polluted areas that increased the frequency of intersexuality in harpacticoid copepods where intersexuality is extremely rare (61), in amphipods (62) and in decapods (63). These findings raised major concerns regarding the impacts of pollutants on the reproductive success of many crustacean species.

## THE IAG-SWITCH—A MASTER SEX CONTROLLING DEVICE IN CRUSTACEANS

As described above, in male crustaceans, the AG is a unique endocrine organ, secreting the *IAG* hormone, which serves as a master universal sex-differentiating switch abundant among crustaceans, thus termed the “*IAG-switch*” (13). A scheme describing the putative location of the *IAG-switch* from genotypic determination to sexual maturation in gonochoristic crustaceans is given in **Figure 1**. Residing within the eyestalk-AG-testis endocrine axis (64), the *IAG-switch* is controlled by upstream neuropeptides and interacts downstream with *IAG* receptors and binding proteins (51, 65–68). The neuropeptides that mediate growth and reproduction are produced in the X-organ (located in the eyestalk) and later accumulated in the adjacent sinus gland, from where they are secreted. It was found that eyestalk ablation in males caused hypertrophy and hyperplasia of the AG (64, 69) as well as over-expression of the *IAG* hormone (70) and of a membrane-anchored AG-specific factor (71). Therefore, it was suggested that some X-organ derived neuropeptides are upstream controlling elements of AG activity (64, 72, 73). Moreover, a reduction in the transcript levels of gonad-inhibiting hormone (GIH), molt-inhibiting hormone (MIH) and other eyestalk-derived neuropeptides (by using RNAi knock down) significantly increased *IAG* expression. On the basis of these findings, it was postulated that these neuropeptides exert an upstream function that controls AG activity (i.e., *IAG* secretion) (73). It has also been suggested that female molting factors, perceived by males via their short lateral antennules (functioning as olfactory organs), also contribute to the regulation of AG function and male gonadal maturation by increasing *IAG* expression, thus implying that the *IAG-switch* is also partially controlled by female reproductive activity (74).

Studying elements that are downstream to the AG within the insulin-like signaling pathway revealed several insulin receptors that interact with the *IAG* hormone. It was found that some receptors were neither sex specific nor tissue specific, but silencing their encoding genes resulted in AG hypertrophy and over-expression of the *IAG* hormone (51). In contrast, other receptors were male specific, and their knock down led to the arrest of most of the germ cells in the testes at the secondary spermatocyte stage (vs. those in the control group, which developed into sperm cells) (68). A study of the downstream signaling pathway of the *IAG-switch* also revealed an insulin-like binding protein (ILBP) that interacted with the *IAG* hormone, but whose expression was not AG specific (65). This finding indicated that the protein was perhaps synthesized in a location other than the AG, a



premise later supported by a study showing that eight ILBPs characterized in a lobster were neither sex specific nor tissue specific (66). Nevertheless, while some studies have demonstrated that eyestalk-derived neuropeptides (e.g., GIH and MIH) are upstream elements to the IAG-switch and insulin receptors are downstream elements, to the best of our knowledge, specific ILBPs associated with the IAG-switch are still to be found.

The pivotal role of the IAG-switch in governing crustacean sexual differentiation has been under study ever since the discovery of the AG and is, in fact, best exemplified by functional experiments that were performed long before the discovery of the IAG. These studies are revisited here to exemplify the pivotal

role of the IAG-switch and its universality among crustacean species (see also a summary of IAG-manipulation experiments performed to date in **Table 2**).

From an historical point of view, the first researcher to manipulate the IAG-switch, following the discovery of the AG in crustaceans (1), was Charniaux-Cotton (2). In her pioneering experiments on the amphipod *O. gammarellus*, she demonstrated that implantation of the AG into females induced the development of masculine characters and inhibited vitellogenesis, whereas the implantation of testicular tissue had no such effect (2, 3, 75). Later, studies on the terrestrial isopod *A. vulgare* showed that AG implantation (76) or injection of AG extract into females (5) induced partial masculinization,

**TABLE 2** | Summary of experiments that included IAG-switch based manipulations in crustaceans.

Species	Order	Method	Results	References
<i>Orchestia gammarellus</i>	Amphipoda	AG implantation in females	Development of masculine characters and vitellogenesis inhibition	(2, 75)
<i>Armadillidium vulgare</i>	Isopoda	AG implantation in females	Transformation of gonads	(76)
<i>Armadillidium vulgare</i>	Isopoda	Injection of AG extracts into immature females	Transformation of gonads	(5)
<i>Carcinus maenus</i>	Decapoda	AG implantation in females	Development of male secondary characteristics	(77)
<i>Lysmata seticaudata</i>	Decapoda	AG implantation in females	Development of male secondary characteristics	(78)
<i>Pandalus borealis</i>	Decapoda	AG implantation in females	Development of male secondary characteristics	(79)
<i>Rhithropanopeus harrisi</i>	Decapoda	AG implantation in females	Development of male secondary characteristics	(80)
<i>Palaemon varians</i>	Decapoda	AG implantation in females	Development of male secondary characteristics	(81, 82)
<i>Macrobrachium rosenbergii</i>	Decapoda	AG ablation in males	Loss of masculine appendages and transformation of gonads	(83)
<i>Macrobrachium rosenbergii</i>	Decapoda	AG implantation in females	Development of masculine appendages and transformation of gonads	(84)
<i>Macrobrachium rosenbergii</i>	Decapoda	AG implantation in females	Full sex reversal of females to males	(20)
<i>Macrobrachium rosenbergii</i>	Decapoda	AG ablation in males	Full sex reversal of males to females	(85)
<i>Macrobrachium rosenbergii</i>	Decapoda	IAG knock-down using RNAi in males	Full sex reversal of males to females	(86)
<i>Macrobrachium rosenbergii</i>	Decapoda	AG cells transplantation in females	Full sex reversal of females to males	(22, 42)
<i>Procambarus clarkii</i>	Decapoda	AG implantation in females	Development of male secondary characteristics	(87)
<i>Procambarus clarkii</i>	Decapoda	AG implantation in females	Development of masculine characters and inhibition of vitellogenesis	(88)
<i>Eriocheir japonicus</i>	Decapoda	AG implantation in females	Development of masculine appendages	(89)
<i>Cherax destructor</i>	Decapoda	Injection of AG extracts into females	Development of male gonopores and inhibition of vitellogenesis	(90)
<i>Cherax quadricarinatus</i>	Decapoda	AG implantation in females	Development of masculine characters and inhibition of vitellogenesis	(91)
<i>Cherax quadricarinatus</i>	Decapoda	AG ablation in male-intersexuals	Loss of male secondary characteristics and induction of vitellogenesis	(92)
<i>Cherax quadricarinatus</i>	Decapoda	AG ablation in male-intersexuals	Loss of mating behavior with females and fighting behavior with males	(93)
<i>Cherax quadricarinatus</i>	Decapoda	IAG knock-down using RNAi in male-intersexuals	Vitellogenesis induction	(94)
<i>Scylla paramamosain</i>	Decapoda	AG implantation in females	Ovarian regression	(95)
<i>Eriocheir sinensis</i>	Decapoda	Injection of AG extracts from <i>S. paramamosain</i> and <i>E. sinensis</i> into females	Development of male gonopods	(96)
<i>Procambarus virginalis</i>	Decapoda	AG implantation from <i>P. clarkii</i>	Development of male secondary characteristics	(97)
<i>Litopenaeus vannamei</i>	Decapoda	AG ablation in males	Loss of masculine appendages and degradation of spermatids in the gonads	(98)
<i>Litopenaeus vannamei</i>	Decapoda	AG implantation in females	Partial development of male secondary characteristics	(99)

including the transformation of female reproductive organs into testes, sperm ducts and seminal vesicles.

Most IAG-switch manipulation experiments were performed in decapod crustaceans, including shrimp, prawns, crayfish and crabs (100). Among the earliest of such experiments in decapods were those performed on hermaphrodite species (100, 101); for example, AG implantation into females of the simultaneous hermaphrodite, the Monaco shrimp *Lysmata seticaudata* (78), and females of the sequential protandric Northern shrimp *Pandalus borealis* (79) resulted in the development of male secondary characteristics. In gonochoristic species, AG implantation caused episodic

development of external male characteristics, as in the green shore crab *Carcinus maenus* (77) and the Harris mud crab *Rhithropanopeus harrisi* (80). In the Japanese mitten crab *E. japonicus*, AG-implanted females developed masculine appendages, even though all of them retained their oviducts (89), but in the mud crab *Scylla paramamosain*, ovarian regression occurred in female crabs implanted with AG (95). IAG-switch manipulations in crabs also suggested interspecies cross-activity of AG factors, as injection of an AG extract from *S. paramamosain* or *E. sinensis* males into *E. sinensis* females resulted in the development of male gonopods (96).

Many of the studies on the IAG-switch have been conducted in different species of crayfish. In *C. quadricarinatus*, implantation of hypertrophied AGs into females resulted in the development of masculine secondary sex characters, such as the typical red patch on the chela, and in a significant reduction in the gonadosomatic index and impairment of vitellogenesis (91). Particularly revealing studies were those conducted on intersex *C. quadricarinatus* animals. In this species, some animals in the population naturally exhibit intersexuality, in which individuals function as males and exhibit male secondary characters, but bear a mix of male and female gonopores and gonads (active testes on one side and a pre-vitellogenic ovary on the other) (102). In AG-ablated *C. quadricarinatus* intersexuals, there was a partial shift toward femaleness in that external male characters were not regenerated, but expression of the vitellogenin gene was induced (92). Moreover, examination of the agonistic and mating behavior of AG-ablated *C. quadricarinatus* intersexuals revealed that these animals did not exhibit typical mating behavior when exposed to females or the usual fighting behavior when confronted with males (93). Knocking down the IAG gene—by using RNAi through injection of dsCq-IAG—to *C. quadricarinatus* intersexuals caused elevation of vitellogenin expression to a level that did not differ from that in intact vitellogenic females (94). In a related species, the common yabby, *C. destructor*, a significant number of females (vs. a control group) injected with AG extract developed male gonopores at the base of the fifth pereopod and showed inhibition of vitellogenesis (90). In some early experiments carried out in the red swamp crayfish *Procambarus clarkii*, implantation of AGs into females led to partial masculinization, as shown by the partial transformation of the first pair of pleopods into typical male gonopods (87) and by the inhibition of vitellogenesis in mature females (88). One of the most peculiar IAG-switch-based manipulations was performed on a crayfish species in which males do not exist—the marbled crayfish *P. virginalis*. This parthenogenetic species, in which a virginal form of reproduction gives rise to identical clones of all-female progeny (103, 104), nonetheless expresses the IAG gene (105). It was thus suggested that *P. virginalis* is a virginal form that diverged from the gonochoristic slough crayfish *P. fallax* (106), a premise supported by a report that both species retain a highly similar sequence of the IAG gene (105). Interestingly, implantation of *P. virginalis* females with AGs from *P. clarkii*, a related species, resulted in the appearance of masculine external characteristics, such as thickening of the first and second pairs of pleopods and the formation of reversed spines on the third and fourth pairs of pleopods (97). The above findings indicate not only that cross-activity of AG factors occurs between species, but surprisingly that in crustacean species, even those in which males do not exist, female animals are still susceptible to the effects of IAG-switch-based manipulations.

Studies on IAG manipulation in species of Palaemonidae have been reported from 1979/80 onwards. Among these, transplantation of AG grafts induced the development of external masculine characteristics in the common ditch shrimp

*Palaemon varians* (81, 82), and the first partial sex reversal using IAG-switch manipulation that was undertaken in *M. rosenbergii*, the most extensively studied palaemonid. In the latter study, most andrectomized males did not regenerate the appendix masculina (AM), and a reduction of spermatogenic lobules in the testes was observed, while some AG-ablated males showed the development of female gonopores and oviducts and even the initiation of oogenesis (83). In a complementary study, it was shown that AG-implanted females developed masculine characters, including AMs and sperm ducts, and in some cases spermatogenesis was initiated in the gonads (84). That study also served to highlight the IAG-switch as a pivotal sex differentiating mechanism in crustaceans, since females implanted with sperm duct or testicular tissue were not masculinized, while ~80% of AG-implanted females showed some degree of masculinization (84). In another important aquaculture species, the penaeid Pacific white shrimp *Litopenaeus vannamei*, AG ablation of males in various post-larval stages resulted in inferior development of the AMs and degradation of the spermatids in the gonads (98), while AG implanted females did not develop AMs and only the minority developed male-like claspers on the first endopods (99). However, we note that complete and functional sex reversal in this important species has not yet been achieved, despite the extensive attempts of various research groups around the world.

While the above studies have indeed demonstrated the crucial role of the IAG-switch in sexual differentiation in several orders and many species in the Crustacea, all the above-described cases of IAG-switch manipulations yielded various types of partial sex shifts but not fully functional sex reversal of one sex into the other. As shown in **Figure 1**, we assume that a putative decision point exists, in which an individual commences toward sexual maturation as a male or a female. It is hypothesized that IAG-switch controlling factors are accommodating this decision point. Functional experiments manipulating such factors will open a new window into IAG-switch upstream controlling mechanism and might achieve a complete shift between sexes. In order to find such IAG-switch controlling factors, advanced next generation sequencing (NGS) techniques may be employed to sequence the RNA of males and females at early developmental stages whose investigation might yield sexually biased genes that putatively control the IAG-switch. Those genes could be manipulated by knockdown techniques such as RNAi (107) or Morpholino oligos (108) and, if performed before the decision point, might lead to a functional shift between sexes. However, timing the decision point is species-specific and body size of the animal in such early developmental stage might be very small which makes the RNAi/Morpholino manipulation complicated. To overcome the size obstacle, a whole genome sequencing of the animal using latest NGS platforms could be used followed by CRISPR-Cas9 genome editing operations (109) that could be performed at the embryonic level and guarantee that the manipulation occurs before the decision point. To the best of our knowledge, CRISPR editing of IAG-switch related factors was never performed. In the next section, successful IAG-switch manipulation resulting with full sex reversal will be described.

## IAG-SWITCH BASED BIOTECHNOLOGIES FOR PRODUCING MONOSEX POPULATIONS

The use of monosex populations is common in animal husbandry, since in many species males and females yield different agricultural products, particularly fish (110–112), poultry (113–115), and mammals (116). In crustaceans, monosex populations offer particular advantages in aquaculture (85, 117–119), since most decapod species exhibit dimorphic growth patterns, leading to variations in animal size at harvest. The dimorphic growth patterns, in turn, could be a result of different growth rates and behavioral patterns (120) and different food conversion ratio (FCR) values between the sexes (121, 122), or even cannibalism (96). Monosex populations of crustaceans can also be exploited in ecological applications, e.g., monosex prawn populations could be used as bio-control agents, serving as predators of the snails that damage rice crops (123) and that are vectors of parasites hazardous to humans (11, 124–127) and fish (128). Here, we should remember that introducing new species as bio-control agents into a given niche may result in devastating consequences to the ecosystem (129), and therefore monosex populations are preferable as biocontrol agents, since they are not able to reproduce and thus become invasive species.

In the exploitation of monosex aquaculture for yield improvement in crustacean species, the choice of sex will generally be guided by the optimal growth rates and size at harvest. Therefore, all-male aquaculture was proposed for species exhibiting male superiority, as is the case for most crayfish (130–132), lobsters (133), prawns (117, 120, 134) and crabs (135), while all-female aquaculture was suggested for shrimp species in which females are larger than males (118, 136). However, growth rates and size are not the only considerations in the choice of sex for monosex cultures; an additional consideration is the desired product: For example, for the edible female gonads of *E. sinensis* (137), the harvested animals would be vitellogenic females with developed ovaries, even if their body size is smaller than males. Additionally, even in some species in which males are larger than females, such as *M. rosenbergii* prawns (85), monosex female culture could improve the yield and profit in two possible ways: intensification of stocking densities permitted by the lack of aggressiveness of the females (138), and elimination of the need for costly size-selective harvests by virtue of the size uniformity of females (117, 139–141).

Traditionally, monosex aquaculture is achieved through manual sorting (117, 142), which is both time consuming and labor intensive and does not guarantee a 100% monosex population. Agro-biotechnologies are thus needed to replace this traditional method. To date, efforts to establish either all-male or all-female populations, for both WZ/ZZ and XX/XY sex heritability schemes, start with an initial sex reversal step of male to a female or vice versa, based on manipulating the IAG-switch during the sex differentiation process (see schemes in **Figure 2** which represent the methodology to achieve all-male and all-female populations in both WZ/ZZ and XX/XY systems). However, all the IAG-switch manipulations performed

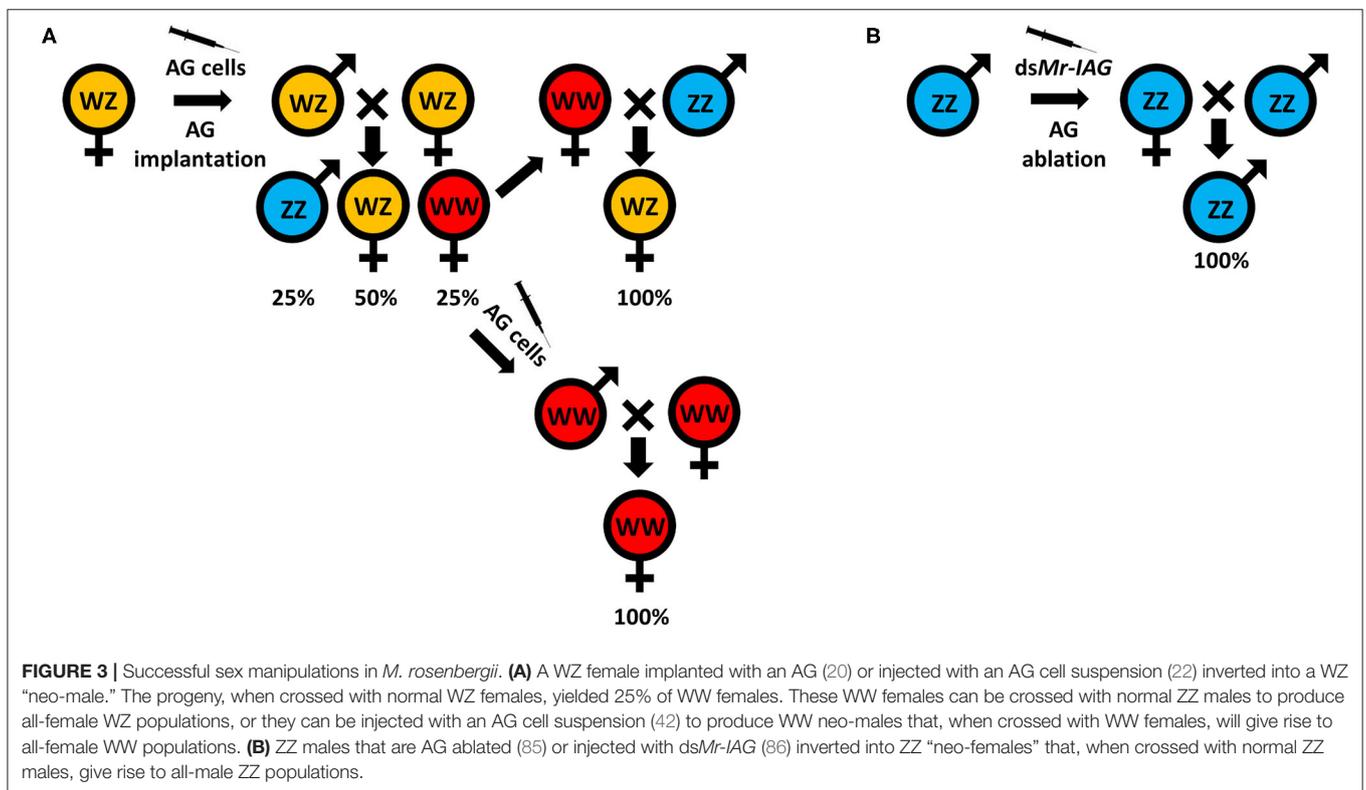
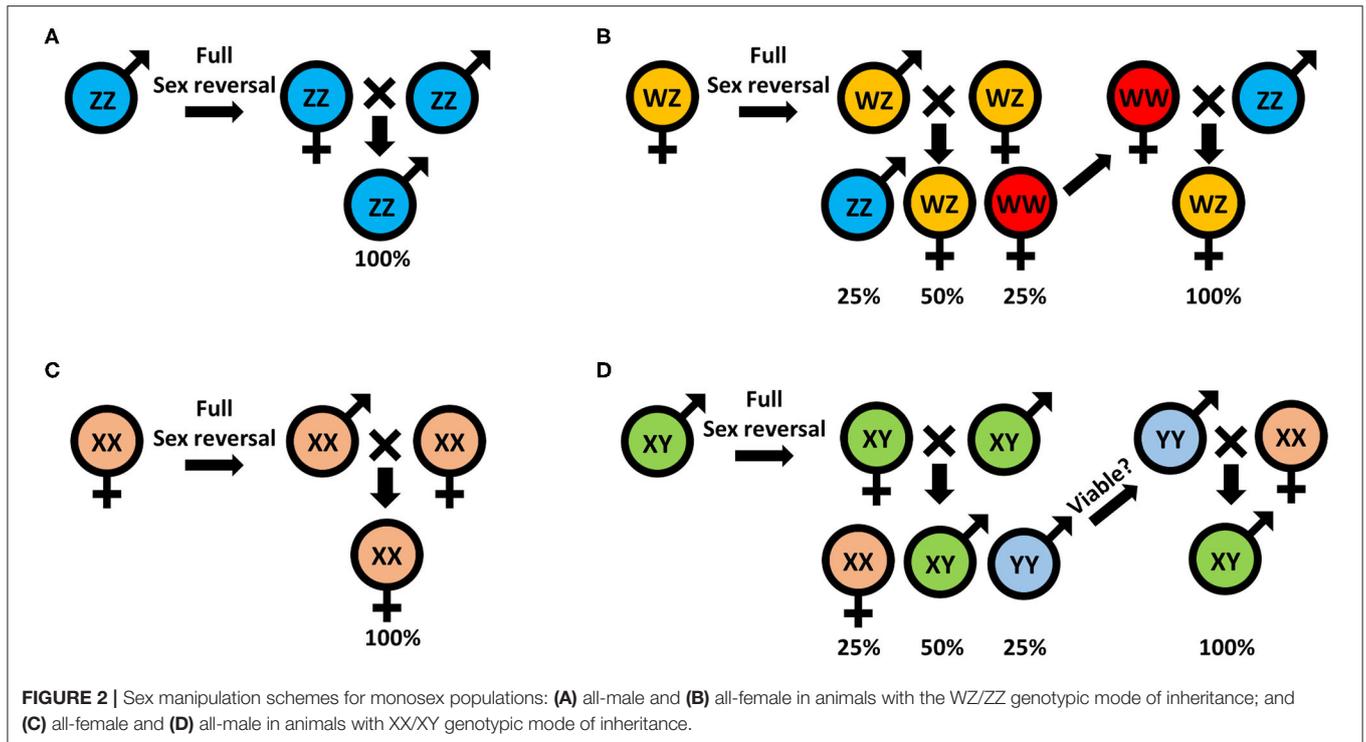
to date have resulted only in partial sexual shifts, with the exception of the fully functional sex reversal in the decapod species *M. rosenbergii* (20, 22, 42, 85, 86). All-female progenies of *M. rosenbergii* were achieved in the following way: Implantation of AGs in juvenile WZ females resulted in sex reversal to WZ “neo-males.” When these neo-males were crossed with normal WZ females, a quarter of the progeny comprised viable WW females. Crossing of the WW females with normal ZZ males produced a monosex WZ female population (20) (**Figure 3A**). In contrast, to produce a monosex ZZ male population, the first step was AG ablation of juvenile ZZ males, which sex reversed them into ZZ “neo-females.” Crossing these neo-females with normal ZZ males produced a monosex ZZ male population (85) (**Figure 3B**). Even though a single sex reversed animal may yield several monosex progenies of thousands of prawns, the complicated surgical procedure of AG ablation/implantation resulted in high mortality and low rates of fully sex reversed animals (20, 85), and the above sex reversal schemes were therefore not suitable for scaling up toward commercialization. The break-through was made with the development of the first RNAi-based biotechnology for *M. rosenbergii* monosex aquaculture, which relied on knock down of the IAG gene through a single injection of ds*Mr-IAG* into ZZ males at an early post-larval stage. This biotechnology successfully enabled mass production of ZZ neo-females and consequently of all-male aquaculture (86) (**Figure 3B**). This procedure has been commercialized and has already yielded several consecutive generations of W-free ZZ prawns (143).

For generating all-female populations, the complicated procedure of AG implantation into females was replaced with a single injection of AG cell suspension into WZ females at an early post-larval stage; this yielded WZ neo-males and subsequently WW females and all-female aquaculture (22) (**Figure 3A**). This biotechnology was later improved by using the same procedure of AG cell transplantation into WW females that were then sex reversed into WW neo-males. Crossing of the WW neo-males with WW females yielded all-female WW progenies, thereby making the production of all-female producing females much more efficient (42). We note that when this procedure was performed repeatedly, it, too, yielded consecutive generations of Z-free WW prawns (144).

The above studies on *M. rosenbergii* yielded both males and females of every possible genotype (ZZ, WZ and WW). This sexual plasticity further highlights the pivotal role of the IAG-switch in crustacean sexual differentiation, since even after the genotype is determined, manipulating the IAG-switch may alter the initial direction of sexual development toward maleness or femaleness, with a probable complete autosomal sex-differentiation toolkit for each gender, regardless of the presence or absence of either sex chromosome.

### WHY INSULIN-LIKE?

The above review of the pivotal involvement of an ILP as a major factor in crustacean sex differentiation demands a broader evolutionary discussion of ILPs in the context of sex



regulation and reproduction in the animal kingdom, in which certain aspects of sexual differentiation are largely controlled by vertebrate-like sex steroids rather than ILPs. Indeed, similar to

the IAG hormone, sex steroids (including androgens, estrogens and progestogens) mediate sexual development, secondary sex characters and dimorphic male/female physiological and

behavioral patterns (145, 146) in most vertebrates, including mammals, birds, reptiles, amphibians and fish (147). In aquaculture, this role for sex steroids finds application in the production of monosex fish populations by sex reversal of females into males or vice versa through  $17\alpha$ -methyltestosterone or estradiol- $17\beta$  administration, as applicable (148–150).

An evolutionary survey of the animal kingdom reveals that vertebrate-type sex steroids (mostly estrogen, androgen and progesterone) and their related receptors and binding proteins are also found in aquatic invertebrates, including different classes of mollusks (151) [such as gastropods (152, 153), bivalves (154, 155), and cephalopods (156–158)] and echinoderms (159) [such as starfish (160) and sea urchins (161)]. Moreover, sex steroids are also found in flatworms (162), annelids (163), crustaceans (164), and cnidarians, such as corals (165). Their function in aquatic invertebrates is believed to be associated with reproduction (but not necessarily with sex differentiation) through the control of the levels of noradrenaline and dopamine, gonadic serotonin and catecholamine, and even cell metabolism and immunity (151). Moreover, as is the case for vertebrates (166), in invertebrates sex steroids are involved in growth processes. In crustaceans, and other arthropods, such growth processes depend on a periodic molt cycle in which the animal sheds its old extracellular cuticle and forms a new, larger cuticle (167). In crustaceans, steroid hormones (ecdysteroids) play a major role in molting and other developmental processes that are regulated by neuropeptides, such as MIH secreted from the Y-organ (168–170). Additionally, in some crustaceans, reproduction is linked to a pre-mating molt, which suggests some sort of coordination between molt-controlling steroid agents and gonad maturation (171). However, while the involvement of steroids in growth processes of crustaceans and other arthropods is clear, their function in controlling reproduction in crustaceans has been called into question. Nevertheless, it has been reported that vertebrate-like sex steroids could be involved in crustacean reproduction, as, for example, administration of progesterone induced ovarian maturation and spawning in the shrimp *Metapenaeus ensis* (172) and vitellogenesis in the shrimp *Penaeus japonicus* (173), while estradiol treatments promoted vitellogenesis in the crab *Portunus trituberculatus* (174). In contrast, administration of testosterone to female *Ocyropa platytarsis* crabs resulted in masculinization of the ovaries (175), and when administered to male *Parapenaeopsis hardwickii* shrimp, it even caused hypertrophy and hyperplasia of the AG (176). Moreover, administration of estradiol to entire populations of the freshwater amphipod *G. pulex* and of the decapod *L. vannamei* resulted in a clear female bias (57, 177). However, to the best of our knowledge, there are no reports of a fully functional sex reversal in crustaceans following the administration of vertebrate-like sex steroids, which implies that a different factor might be the main regulator in crustacean sexual differentiation. As described above, it is likely that an ILP, namely, the IAG hormone, is such a controlling element. Nonetheless, questions regarding the involvement of an insulin-like factor, rather than a steroid, as the master sex controlling switch in crustaceans, and possible interactions between ILPs and steroids remain open.

As a step toward addressing these questions, let us examine the insulin superfamily. This group of proteins includes ILPs with a typical proteomic structure of B and A chains linked by disulfide bonds (178). Peptides of the insulin family are found in protozoans (179) and metazoans—both vertebrates and invertebrates (180). ILPs were first discovered in mammals and attracted extensive interest due to their involvement in many physiological processes (181). In vertebrates, they comprise a set of proteins including insulin, insulin-like growth factors (IGFs) and relaxins, which are essential in reproduction, growth, and developmental and metabolic pathways, such as carbohydrate and lipid metabolism (182–185).

In invertebrates, the first ILP was found in the clam *Mya arenaria* (186), and since then such proteins have been found in many species across different classes, including mollusks (187), annelids (188), flatworms (189), cnidarians (190), sponges (191), nematodes (192) and arthropods (193). A regulatory interaction between ILPs and steroids has indeed been found in insects in which the prothoracicotropic hormone (PTTH), a brain neuropeptide, controls the secretion of the ecdysteroids that regulate molting (194). Bombyxin, such a PTTH found in the silkworm *Bombyx mori* was found to be homologous to insulin (195). In addition to its effect on growth (196) and cell proliferation (197) in lepidopterans, bombyxin is also involved in ovarian development in dipterans (198). ILPs are also found in orthopterans, such as the migratory locust *Locusta migratoria*, in which a single copy of an ILP is expressed as two transcripts; one serving as a putative neurohormone is expressed in the brain, and the other serving as a putative growth factor is not tissue specific (199). In addition to their structural resemblance at the protein level, the conservation of invertebrate ILPs within vertebrates is best exemplified by the fact that an insulin-like protein extracted from the common fruit fly, *D. melanogaster*, showed cross reactivity between species by initiating insulin bioactivity in mice (200), while mammalian insulin was successful in activating *D. melanogaster* insulin receptors (201). Moreover, injection of recombinant human insulin into the shrimp *L. vannamei* led to increased levels of glucose in the hemolymph and of glycogen in the gills, thus suggesting that ILPs play a role in crustacean carbohydrate metabolism (202). Crustaceans are also known to possess ILPs that serve as growth factors (65) and some that regulate glucose metabolism and participate in the immune response against pathogens (203). However, while ILPs are generally not regarded as sex specific, crustaceans constitute a unique group in which a male-specific ILP (the IAG hormone) is the master factor in regulating sexual differentiation (8). This function of an ILP raises questions regarding the speciation of insulins into a variety of different physiological pathways during evolution, especially during the shift from invertebrates to vertebrates. However, while crustacean ILPs and vertebrate-like steroids share a physiological function as growth factors, the evolutionary processes regarding the alteration of the major sex differentiating mechanism from ILPs in early pancrustaceans (i.e., the crustacean IAG-switch) to sex steroids in vertebrates are still unknown.

## CONCLUSIONS

The process of crustacean sexual development from the genotypic sex determination (WZ, ZZ, XX or XY), through the sexual differentiation process, to the final masculine or feminine maturation involves various sex controlling mechanisms that include factors such as ILPs or steroids. The IAG-switch is a unique crustacean endocrine-controlling mechanism involving an ILP that regulates sexual differentiation and function within the eyestalk-AG-testis endocrine axis. Despite earlier determination of the sexual genotype, the switch can be manipulated to induce either masculinization or feminization, thereby revealing striking sexual plasticity in crustaceans. It is this sexual plasticity that is often being exploited for sex manipulations for the establishment of monosex populations.

During the evolution of ILPs in the animal kingdom, numerous functions have evolved for such proteins in both invertebrates and vertebrates. A unique ILP function that evolved in the Crustacea is the IAG-switch mechanism, which

constitutes the pivotal element in the sex differentiation processes. Nevertheless, to reveal the evolutionary pathways of sex differentiating controllers, i.e., ILPs in crustaceans and sex steroids in vertebrates, further evolutionary studies focusing on sex differentiation during the evolution of arthropods and the shift from invertebrates to vertebrates are required.

## AUTHOR CONTRIBUTIONS

This manuscript was conceived and written by TL and AS. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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