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A Novel Hormone Is Required for the Development of Reproductive Phenotypes in Adult Female Crabs

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The crustacean male-specific androgenic hormone is widely accepted as a key factor in sexual differentiation and in the development of secondary sex characteristics. However, the mechanism by which the plethora of different reproductive strategies are controlled and executed in crustaceans is not known. We discovered in the blue crab, *Callinectes sapidus*, a hitherto unknown neurohormone, named crustacean female sex hormone (CFSH), in distinct neurosecretory cells in the eyestalk ganglia. CFSH is highly expressed in females but weakly in males, and its crucial role in developing adult female phenotypes has now been established. *CFSH* cDNA encodes a 225-amino acid (aa) novel protein composed of a 23-aa predicted signal peptide, 33-aa precursor-related peptide and 167-aa mature protein that did not match any other sequence in GenBank. *CFSH* RNA interference knockdown by multiple administrations of double-stranded RNA at the prepubertal stage causes abnormal development of brooding and mating systems upon puberty. These systems include a pair of gonopores and an egg attachment system for brooding, comprised of an enlarged semicircular abdomen and ovigerous setae. The ovigerous setae in *CFSH* knocked-down females were fewer and 50% shorter and the gonopores were either significantly smaller than those of controls, misplaced, or absent. We also identified CFSH in the green crab, *Carcinus maenas*, a species that shares a similar reproductive strategy with *C. sapidus*. Together, our data provide the first evidence for the presence of a female hormone in crustaceans and its importance in positively controlling anatomic features associated with brooding and mating systems. From an evolutionary standpoint, the endocrine control supporting a female-specific reproductive strategy, as previously described for many vertebrate species, has now been demonstrated for the first time in crustaceans. **(*Endocrinology* 154: 0000–0000, 2013)**

Sexual selection in most gonochoristic animals drives the development of sex-specific secondary features that support the species' unique reproductive strategies. In many vertebrates and some invertebrates, parents provide parental care of offspring to promote the survival and well being of the next generation at a cost to the resources of the current (1, 2). Most processes rely on changes initiated and maintained by key reproductive hormones. In mammals, for example, peptide hormones and steroids secreted during pregnancy prime and regulate the physiology and maternal care behavior (3, 4). In insects, juvenile hormone (JH) regulates the trade-off between care for current offspring and the production of new eggs/clutches (5–7).

In crustaceans, morphologic features specific to adults, particularly those of females, develop at the pubertal or pubertal-terminal molt, known also as a partial metamorphic molt (8). Removal of the male androgenic gland or silencing of its major product, the androgenic gland hormone (AGH), results in feminization of males in several crustacean species. Hence, it is widely accepted that AGH, also termed insulin-like androgenic hormone (IAG), plays a critical role in male differentiation and the development of secondary male characteristics (9). The development of

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Abbreviations: aa, amino acid; AGH, androgenic gland hormone; CFSH, crustacean female sex hormone; CHH, crustacean hyperglycemic hormone; dsRNA, double-stranded RNA; GFP, green fluorescent protein; h, height; IAG, insulin-like androgenic hormone; JH, juvenile hormone; MIH, molt-inhubiting hormone; MTXO, medulla terminalis X-organ; nt, nucleotide; RACE, rapid amplification of cDNA ends; rCFSH, recombinant CFSH; RP-HPLC, reversed phase HPLC; SG, sinus gland; UTR, untranslated region; w, width.

phenotypic females is assumed to be a "default," in the absence of AGH (10). However, this limited endocrine framework seems insufficient to provide the hormonal support for the vastly diverse reproductive strategies that are found among crustaceans.

Females of crustacean species that exhibit soft-shell mating immediately after the pubertal or pubertal-terminal molt are equipped with unique adult morphologic features specialized for mating and brooding, which may develop gradually before or at puberty (11). Similar to other crab species, such as the edible crab, *Cancer pagurus* (13) and the snow crab, *Chionoecetes opilio* and *C. baridi* (14), most *Callinectes sapidus* females experience a single mating during their lifetime immediately after their pubertal-terminal molt (8, 12), which renders them capable of spawning multiple times using stored spermatophores and carrying a full-sib brood (12). These females invest in 1) a brooding system that includes an egg-attachment system comprised of numerous hair-like ovigerous and plumose setae on the pleopods and an enlarged semicircular abdomen with a large capacity of up to 8 million embryos that appear at the pubertal molt; 2) a mating system that includes enlarged pairs of gonopores for sperm transfer and spermathecae for long-term sperm storage that develop gradually before puberty (15–17). Importantly, in *C. sapidus* females, the ovigerous and plumose setae comprising the egg attachment system, must be completely replaced with a new set of setae at each spawning event (up to 18 times) during the span of adulthood (8, 18, 19).

In some crustacean females exhibiting the pubertal-terminal molt, such as the spider crab (*Libinia emarginata* (18)) and *C. sapidus* (our observation), eyestalk ablation of the females at prepuberty results in impaired development of mating and maternal care structures. Eyestalkless-females do molt and become giant immature crabs (20), devoid of the structures specific to adults that are required for successful mating and brooding (21). These observations support the notion that the development of these adult-specific morphologic features requires an input(s) from the eyestalk ganglia. Therefore, we aimed to identify, and determine the role of this factor responsible for the development of these secondary sexual structures.

We hypothesized that if an endocrine source(s) was responsible for the development of adult-specific features supporting brooding i*n C. sapidus*, it may originate in the eyestalk ganglia. Herein, we describe a novel hormone (crustacean female sex hormone [CFSH]), predominantly expressed in female eyestalk ganglia, and its integral role in the regulation of the pubertal-termimal molt morphogenesis of reproductive structures in *C. sapidus* females. We also show the presence of CFSH in another crab species that displays a similar reproductive strategy. Our studies demonstrate that a female hormone actively contributes to the development of adult-specific features required for brooding and mating systems.

Materials and Methods

Animals

Young juvenile *C. sapidus* crabs (20–30 mm carapace width) were obtained from the blue crab hatchery (Institute of Marine and Environmental Technology [IMET], Baltimore, MD) and raised in similar conditions as described (22). Adult male and female green crabs, *Carcinus maenas,* were obtained from the Gulf of Maine and kept in 30 ppt seawater until dissected.

Identification and isolation of crustacean female hormone by reverse phase HPLC (RP-HPLC)

CFSH was initially identified in the RP-HPLC chromatograms of isolated single sinus gland (SG) extracts from *C. sapidus* females. Sinus glands of mature males and mature females were excised from the eyestalk ganglia, and peptides were extracted and resolved using RP-HPLC as described below. To isolate the CFSH peptide, extract of 6 pooled SGs (from 3 mature females) was prepared by homogenization using a sonicator with a 2-mm probe (Soniprep) in ice-cold 2 M acetic acid for 10 × 1-second pulses. The preparation was centrifuged at 15 000 × *g* for 10 minutes at 4°C, and the supernatant was dried using vacuum centrifugation and resuspended in 50% acetonitrile before HPLC separation. CFSH had a retention time of 26–27 minutes on a C18 column (Jupiter, Phenomenex) under a gradient of 50%–65% B (A = 0.11% trifluoroacetic acid in 100% water; B = 0.1% trifluoroacetic acid in 40% water and 60% CH_3CN) over 35 minutes at a flow rate of 0.5 mL^{-1}. The collected fraction of CFSH was further resolved on a 4%–20% gradient SDS-PAGE (Bio-Rad Laboratories), and electroblotted to a polyvinylidene difluoride membrane. The single observed band of approximately 20 kDa was excised for N-terminal Edman sequencing in a PerkinElmer/Applied Biosystems Precise Protein Sequencing System (The Synthesis and Sequencing Facility, Johns Hopkins University, Baltimore, Maryland). The same procedure was employed to obtain the N-terminal Edman sequencing of the CFSH of the green crab, *C. maenas*.

Cloning of *C. sapidus* and *C. maenas* CFSH cDNA

Total RNA was extracted from the eyestalk ganglia of a mature female using TRI_{ZOL} reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was generated with 1 μg total RNA using 5′- and 3′-rapid amplification of cDNA ends (RACE) using a SMART RACE cDNA Amplification Kit (BD Biosciences). All primers are listed in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. First PCR amplification was conducted with 3′-RACE cDNA and Advantage Taq polymerase (BD Biosciences) with primers CFSHdF3 and the universal primer (UPM). PCR conditions were: 94°C for 2.5 minutes, 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute. A nested PCR was performed using primer CFSHdF1and the nested universal primer under the same conditions, except for annealing at 45°C. An amplicon of 490 bp

was cloned into a pGEM-T Easy vector (Promega Corp.) and sequenced. 5′- and 3′-untranslated region (UTR) sequences were obtained using 5′- or 3′-RACE cDNA with universal primer and CFSHGSR1 or CFSHGSF1, respectively, and nested amplification with CFSHR2 primer or CFSHF2 and nested universal primer using a GeneAmp High Fidelity PCR System (Applied Biosystems) at 60°C annealing. The 5′-amplicon of 927 bp contained the entire *CFSH* cDNA.

The same cloning strategy was employed to obtain the C*FSH* cDNA of the green crab, *C. maenas*. Because Edman N-terminal sequencing of approximately 18-kDa band isolated from the female SGs resulted in the identical amino acid (aa) sequence to that of the *C. sapidus*, a PCR amplification strategy with degenerate primers at the same PCR conditions that was used to amplify the *C. sapidus CFSH* was adopted.

The sequence homology was examined using the BLAST and Swiss Prot network servers.

Spatial distribution of CFSH expression in *C. sapidus*

Tissue distribution of *CFSH* expression was examined using an end point RT-PCR assay with cDNAs of various tissues from a mature female at initial pubertal stage of ovarian development and a mature male. One microgram of total RNA after DNase I treatment (Fermentas) was reverse transcribed with random hexamer primers and Moloney murine leukemia virus-reverse transcriptase (Promega). Each cDNA template containing 25 ng total RNA, was amplified with primers CFSHF and -R or arginine kinase (AKQF and -QR) and a high fidelity, MyFi DNA polymerase (Bioline) in PCR conditions: 95°C for 1 minute followed by 35 cycles of 95°C for 20 seconds, 62°C for 20 seconds, and 72°C for 20 seconds. PCR products were resolved on 1.5% agarose gel and visualized using ethidium bromide.

Production of recombinant CFSH in *Escherichia coli* for antibody production

CFSH that was amplified using a high-fidelity Taq (BD Biosciences) with primers CFSHExpF1 and CFSHExpR1 containing 2 restriction enzyme sites, *Nco*I and *Xho*I, was cloned into a pET-15b vector and transformed into Rosetta-gami B(DE3)pLysS cells (Novagen). A similar protocol as described by Chung et al (23) was carried out. In brief, the expression of recombinant CFSH (rCFSH) was induced at approximately 0.5 OD_{600} with 1 mM isopropyl-β-D-thiogalactopyranoside, and cells were further grown for 3 hours at 37°C. Cells were harvested by centrifugation at 10 000 $\times$ *g* for 10 minutes at 4°C and washed with PBS. After repeating the previous centrifugation step, pelleted cells were sonicated in ice-cold PBS and centrifuged at 15 000 $\times$ *g* for 10 minutes at 4°C. The protein concentrations of the resulting supernatant and pellet were determined, and the samples were then resolved by 15% SDS-PAGE and visualized with Coomassie Blue staining (Supplemental Figure 1).

For antigen production, the insoluble pellet from 1 L bacterial culture was resolved by 15% SDS-PAGE, and the induced protein with a molecular weight of approximately 18 kDa was excised from the gel. The gel slices containing rCFSH were directly used for antiserum production (ProteinTech Group). The final-bleed antiserum was used in all immunostaining and Western blot applications.

CFSH knockdown using CFSH-double-stranded (ds)RNA in *C. sapidus* females

CFSH-dsRNA, was generated using TranscriptAid T7 High Yield Transcription kit (Fermentas) from 1 μg template of the coding region of mature *CFSH* (399 bp) amplified with an adult female eyestalk cDNA and primers CFSHdsF and- R. For a control, dsRNA-green fluorescent protein (GFP) of 400 bp was amplified from the vector pEGFP-1 (GenBank U55761) with primers GFPdsF and -dsR. Juvenile animals (20–30 mm carapace width) were injected twice a week with 10 μg *CFSH-dsRNA* in 100 μL crustacean saline. Control groups received 100 μL of either 10 μg *GFP-dsRNA* or saline. Injections were made with an insulin syringe (Becton Dickinson) with a 28-gauge needle into the arthrodial membrane located between a chela and the first walking leg. These injection regimes were continued for about 3 months until the animals reached adulthood. Adult-specific morphologic features were compared with those of mature females that were eyestalk ablated at prepuberty (90–100 mm carapace width).

Quantification of CFSH expression in the eyestalk ganglia by quantitative RT-PCR assay and the protein levels of CFSH by Western blot analysis

We measured the levels of both *CFSH* in the neurosecretory cells located within the eyestalk ganglia and of CFSH protein stored in the SG in the same eyestalk ganglia. Total RNAs were extracted from the eyestalk ganglia of *C. sapidus* females of the following experimental groups injected with saline, *GFP-dsRNA*, or *CFSH-dsRNA*. The levels of *CFSH* expression in the sample cDNAs (25 ng total RNA) were assayed in duplicate using SYBR Green PCR mix (Applied Biosystems) with CFSHQF and -QR primers on ABI Prism 7500 Sequence Detection System (Applied Biosystems). For a reference gene, the expression levels of molt-inhibiting hormone (MIH) in the same cDNA samples were determined with MIHQF and -QR primers. Standards for each gene were generated using sense RNA transcribed by RNA polymerase from the cDNA clones (22). The C_T value for each gene was converted to mRNA copy number and normalized against the copy number of *AK* in the corresponding sample.

For SDS-PAGE and Western blot analyses, a single SG (n = 3/each treatment group), homogenized by sonication in 25 μL PBS was denatured in SDS-PAGE sample buffer (Bio-Rad) and 10 μL (= 20% of a SG) was separated by 15% SDS-PAGE. Western blot analysis was employed as described elsewhere (22), with anti-CFSH serum (1:5000) and a Super-Signal West-Pico chemiluminescence detection kit (Pierce Chemical Co.) and exposed to a BioMax MS Kodak film.

Localization of CFSH in the eyestalk using whole-mount immunohistochemistry

Eyestalk ganglia dissected from ice-cold anesthetized crabs and embryos at hatching stage (24) were fixed in a *Drosophila* fixative (4% paraformaldehyde and 7% picric acid in 0.1 M phosphate buffer, pH.7.4) overnight at room temperature and were processed as described elsewhere (24). For double-labeled stainings, we used anti-crustacean hyperglycemic hormone (CHH) and MIH sera that were previously characterized and validated as described (25, 26). The tissues/embryos were incubated overnight with anti-CFSH serum (1:1000) followed by

incubation with secondary antibody (Alexa Fluor 488-goat-antirabbit IgG, Invitrogen) diluted 1:100. These preparations were then washed and incubated with MIH/CHH primary antibodies conjugated to Alexa Fluor 555 (Zenon, Invitrogen) at a final dilution of 1:1000 in PBS-Tween 20. After washing, the preparations were examined and Z-stack images were collected using a Bio-Rad confocal microscope. The colocalization and projection of images was generated using LaserSharp2000 (Bio-Rad).

Statistical analysis

All data were examined using the Kolmogorov-Smirnov test for normality (InStat) prior to all statistical tests. Statistical significance of the data ($P < .05$) was determined using one-way ANOVA with post hoc Tukey-Kramer multiple comparison test (GraphPad, InStat3). The results are presented as mean ± SE (n = 7–13).

Results

Identification of the CFSH in female SGs

CFSH was initially discovered as an additional neuropeptide in extracts of adult female SGs. RP-HPLC analyses of the neuropeptide profile of a single SG from adult female and male *C. sapidus* (Figure 1, A and B, respectively) bore quantitative and qualitative differences in the neuropeptide profiles between the 2 sexes. A notable peak, corresponding to CFSH, is detected only in female SG (Figure 1A), while being absent in the male (Figure 1B). The remaining peaks corresponding to 1) CHH-precursor-related peptide; 2) and 3) isoforms of CHH-1 and -2, respectively; and 4) MIH (25, 27) are consistent in both sexes. The HPLC peak containing CFSH from the pooled female SGs (n = 6) was resolved as a single protein band with a molecular weight of approximately 20 kDa by SDS-PAGE analysis (Figure 1A, inset). This protein band was electroblotted to a polyvinylidene difluoride membrane followed by N-terminal Edman sequencing, which yielded a 20 aa N-terminal sequence: SSIIGHMNSIPYRTREQVMD, corresponding to the N terminus of the mature CFSH, as specified below.

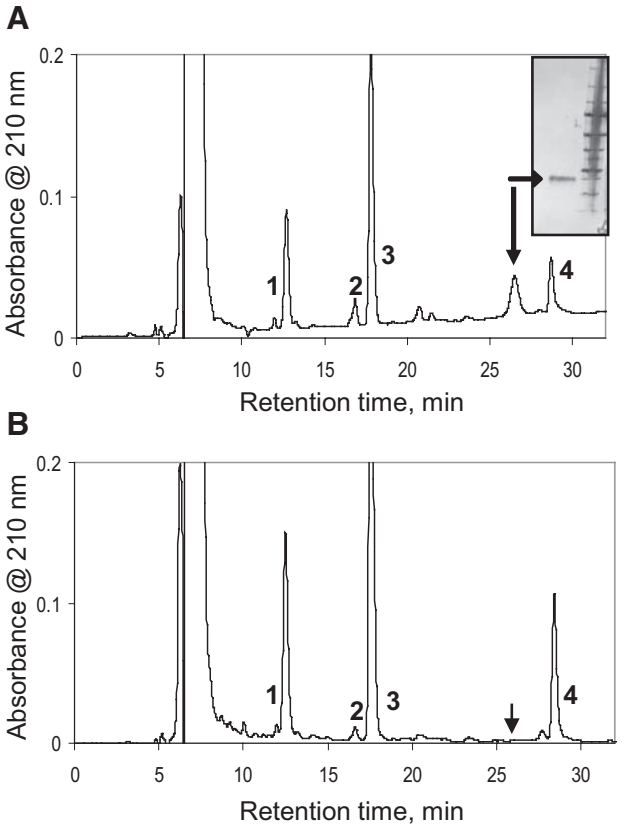

Figure 1. Identification and isolation of CFSH A, Identification of CFSH by RP-HPLC separation of neuropeptides from representative single SGs of an adult female (A and B) and a mature male. The arrows point to CFSH peptide eluted at 27 minutes in the female SG and to its absence in a male SG. Peak 1, CHH-precursor-related peptide; 2, CHH-1; 3, CHH-2; 4, MIH. A inset, Stained polyvinylidene difluoride (PVDF) membrane after protein transfer from the SDS-PAGE separation of the eluted CFSH collected manually from reduced pressure HPLC. The band containing the CFSH, marked with an arrow, was excised from the membrane and analyzed by N-terminal Edman sequencing.

Isolation of the full-length cDNA of CFSH and the deduced CFSH protein

The full-length cDNA of the *CFSH* was cloned from a female eyestalk ganglion using the degenerate primers, which were generated based on the aa sequence obtained from N-terminal Edman sequencing (SSIIGHMNSIPYRTREQVMD). The full-length nucleotide (nt) sequence of *CFSH* included a short 24-nt 5′-UTR, 598-nt open reading frame, and 224-nt 3′-UTR (GenBank GU016328) that encodes a predicted 225-aa novel protein. CFSH appears to be synthesized as a preprohormone (schematic diagram in Figure 2B) comprised of, in order, a 23-aa signal peptide (SignalP, italicized in Figure 2A), 33 aa of CFSH precursor-related peptide, a dibasic cleavage site (KR, predicted by ProP.10), and 167-aa of the mature CFSH. The first 20 deduced aa residues of the predicted mature CFSH are identical to those obtained from the initial N-terminal sequence, thereby confirming Lys and Arg as the dibasic cleavage site. Figure 2B shows 8 cysteine residues, numbered and predicted to form 4 putative intramolecular disulfide bridges: C_{46}-C_{150}, C_{80}-C_{112}, C_{105}-C_{119}, and C_{107}-C_{148} (http://disulfind.dsi.unifi.it). The molecular mass of CFSH is calculated as more than 18731.8 Da with 3 potential phosphorylation sites at T_7, S_{18}, and Y_{23} (NetPhosK) and one O-glycosylation site at S_{47} (OGPETv1.0). The homology searches, Conserved Domain Architecture Retrieval Tool (NCBI) and Swiss Prot did not yield the identification of similar proteins or proteins exhibiting a similar sequence to date.

In order to verify that CFSH is not exclusive to *C. sapidus* females, we examined the adult females of another brachyuran species, the European green crab (*Carcinus*

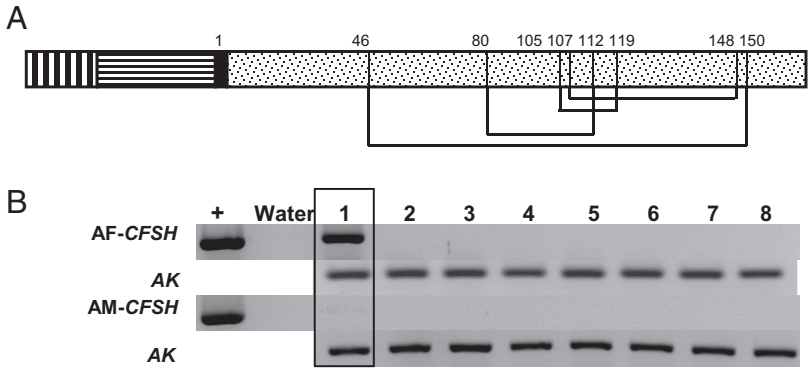

Figure 2. Characterization of *CFSH* cDNA of *C. sapidus* and its spatial expression. A schematic diagram of prepro-CFSH-signal peptide (vertical bar), CFSH-precursor-related peptide (horizontal bar), a dibasic cleavage site (closed bar), and the mature CFSH (dotted bar). The 8 cysteine residues are predicted to form 4 putative disulfide bridges connected with lines. B, Spatial distribution of *CFSH* mRNA generated by PCR on cDNAs from various tissues of an adult female (AF) and male (AM) and compared with arginine kinase (*AK*) gene: 1) ES (boxed), 2) brain, 3) thoracic ganglia, 4) pericardial organ, 5) mandibular organ, 6) Y-organ, 7) gill, and 8) hepatopancreas. Two controls were shown with the amplication of a *CFSH* clone (+) and water (negative).

maenas) using the same strategy. A similar band was identified with RP-HPLC separation of SG neuropeptides and yielded the identical N-terminal sequence. Hence, with the same cloning procedure, a partial sequence of the *C. maenas CFSH* was isolated. The deduced *C. maenas* CFSH sequence shows 90% and 88% sequence identity to that of the *C. sapidus CFSH* at the aa and nucleotide levels, respectively (Supplemental Figure 3).

Spatial expression profiles of *CFSH* in different tissues of a mature *C. sapidus* female and male, generated using an end-point RT-PCR assay, revealed a strong band of 401 bp PCR product corresponding to *CFSH* in female eyestalk cDNA (Figure 2C) and a significantly weaker band in male eyestalk cDNA. The levels of the ubiquitous AK expression, which served as an internal housekeeping control gene, remained similar in all tested tissue cDNAs. This result suggests that the eyestalk is the only site for *CFSH* expression.

Neuroanatomical localization of CFSH in the eyestalk ganglia

Neuroanatomic localization of CFSH protein in the eyestalk ganglia (medulla terminalis X-organ and sinus gland complex [MTXO-SG], Figure 3A) was carried out using whole-mount immunohistochemistry with the anti-CFSH serum generated against the rCFSH. The specificity of the antibody was tested by whole-mount immunohistochemistry on female eyestalks using serum preabsorbed with rCFSH (Figure 3B, inset). Moreover, anti-CHH and MIH sera, the specificities of which have been previously characterized (25, 26), stained different perikarya and did not cross-react with CFSH (Figure 3). Anti-CFSH serum specifically recognized a cluster of 30–50 perikarya, at a size of 25–35 μm per perikarya, as CFSH-producing cells in adult female X-organs (Figure 3, B and D, green) that did not show staining with the incubation of anti-CFSH preabsorbed with rCFSH (Figure 3B, inset). Juvenile females at the 11th or 13th crab stage (30–50 mm carapace width) had a similar number of CFSH cells in the MTXO, but the cells were smaller (~15–20 μm) than those found in mature females (data not shown). These cells are distinct from the cells expressing CHH (Fig. 3B, red) and MIH (Fig. 3C, red), yet their size and staining intensity are comparable to those of CHH and MIH perikarya. In addition, CFSH immunostaining in females is also detected in both the axonal tract, which transports CHH neuropeptides to their storage and release site in the SG, as well as in the SG itself. CFSH staining in the axonal tract is as strong as that of CHH (Figure 3E), the major neuropeptide in the SG, but is reduced in the SG compared with CHH (Figure 3, B and E). This relatively low intensity of CFSH staining in the SG, compared with that of CHH and MIH, is consistent with the ratio of the neuropeptide levels in the SG RP-HPLC profile (Figure 1A). Some embryos at the hatching stage showed CFSH-positive staining in the SGs (Figure 3H). The adult male eyestalk ganglia complex revealed a faint CFSH immunostaining comprised of 2–4 clusters of 5–10 small perikarya, sized 4–6 μm per perikarya (Figure 3, F and G), and almost no staining in the axonal tract (Figure 3, F and F′).

CFSH knockdown using dsRNA

In order to examine the functional role of CFSH in *C. sapidus* females, a loss of function study using RNAi (dsRNA) was employed. To this end, an experimental treatment regime was initiated with juvenile females at the 9th–11th crab stage (carapace width 20–30 mm) and continued until the pubertal-terminal molt. The females were injected biweekly with *CFSH-dsRNA*, whereas the controls received *GFP-dsRNA* or saline. The knockdown effect of *CFSH* mRNA in the neurosecretory cells located in the MTXO and CFSH in the SG, including its efficiency and specificity, was determined in these females by quantitative RT-PCR assay and by Western blot analysis, respectively.

CFSH-dsRNA administration specifically and significantly reduced *CFSH* levels. Females receiving *CFSH-*

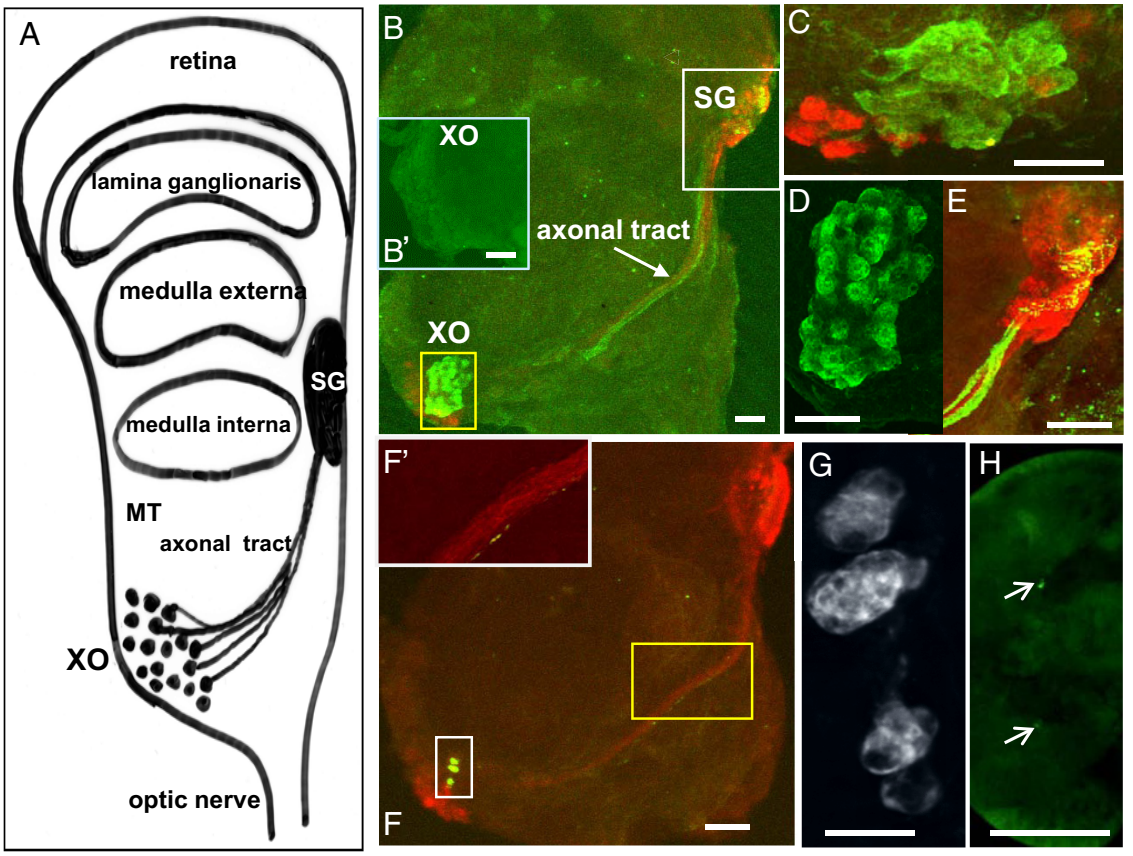

Figure 3. Whole-mount immunohistochemical localization of CFSH in the eyestalk ganglia A, Schematic drawing of a crustacean eye-stalk ganglia containing the MTXO and the interspersing axonal tract. B, Double staining of eyestalk ganglia of an adult female with anti-CFSH serum (green) and anti-CHH serum (red), preabsorption control of XO (B′, inset). C, The same as B but with anti-MIH serum (red). D, Enlargement of CFSH-XO from B (yellow box in B). E, Enlarged axonal tract and SG from A (white box). F, Double staining of CFSH (green) and CHH (red) in male eyestalk. F′, Enlarged image of the axonal tract from F (yellow box). G, Enlargement of the CFSH cell clusters (green) in male XO (white box in panel F). H, CFSH staining in the SG (arrows) of a hatching stage embryo. Scale bar, 50 μm for all plates.

dsRNA contained $0.7 \pm 0.1 \times 10^5$ copies of *CFSH*/μg total RNA, which was approximately 8.5 and 13% of those treated with saline ($5.3 \pm 1.8 \times 10^5$ copies/μg total RNA) or *GFP-dsRNA* ($8.3 \pm 2.8 \times 10^5$ copies/μg total RNA), respectively. *MIH* transcript levels served as a specificity control and remained consistent in all experimental groups (Figure 4A). Untreated mature male MTXOs had low levels of *CFSH* mRNA ($0.8 \pm 0.1 \times 10^5$ copies/μg total RNA), similar to those of *CFSH-dsRNA* treated females. SDS-PAGE (Figure 4B, upper panel) and Western blot analysis (Figure 4B, lower panel) revealed that CFSH is undetectable in the SGs of the *CFSH-dsRNA* treated females (lane 2), as in the males (lane 3), and is detected only in the SGs of the saline-injected control female (lane 1).

In *C. sapidus* females, the morphologic features for brooding are designed to accommodate mating and large brood clutches of 0.75 to 8 million embryos (Supplemental Figure 2). These features include a semicircular abdomen with numerous hair-like ovigerous and plumose setae on 5 pairs of pleopods that hold the embryos during brooding, as well as enlarged gonopores for mating and spermathecae for sperm storage (Figures 5Bb and Supplemental Figure 2A). The most striking phenotypic differences between *CFSH-dsRNA* and *GFP-dsRNA* or saline-treated females were both the size, position, and presence of the gonopores and the development of setae. All females receiving *CFSH-dsRNA* developed abnormal gonopores; 54% of treated females formed a pair of significantly ($P < .001$) smaller gonopores on the sixth sternite (width [w], 2.6 ± 0.3 mm; height [h],1.0 ± 0.1 mm; n = 7) (Figure 5Bf), compared with control females that displayed the normal appearance of the gonopores (w, 3.8 ± 0.3 mm; h, 1.9 ± 0.2 mm; n = 13) (Figure 5Bd). However, their size was similar to the eyestalkless females (w, 2.3 ± 0.2 mm; h, 1.0 ± 0.2 mm; n = 4) (Figure 5Be). Interestingly, of the other 46%, half of these females had gonopores incorrectly placed (Figure 5Bg, n = 3), and the remaining half exhibited no gonopores (Figure 5Bh, circled; n = 3). Similar to eyestalkless animals (Figure 6B), 100% of all females (n = 13) treated with *CFSH-dsRNA* displayed significantly ($P < .001$) impaired development of the embryo-carrying ovigerous setae (Figure 6F), but not of

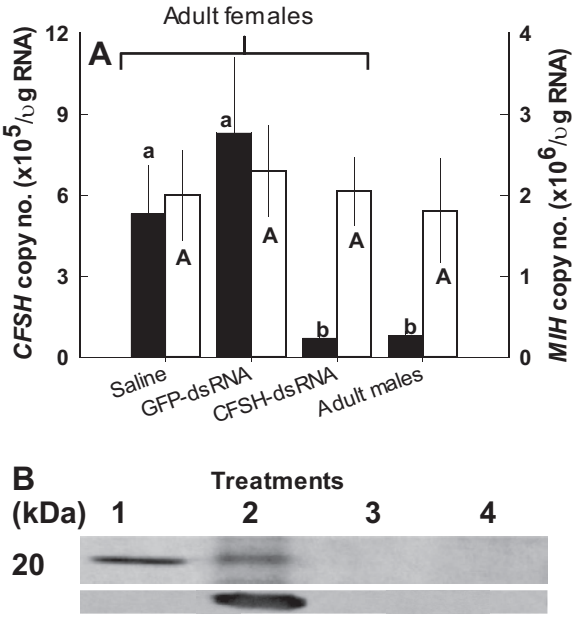

Figure 4. *CFSH-dsRNA* specifically knocks down CFSH A, mRNA levels of *CFSH* (solid bars) and the *MIH* reference gene (open bars) measured from *GFP-* and *CFSH-dsRNA*-treated females and untreated males. The data are presented as mean ± SE (n = 7–13) with different letters indicating statistical significance at $P < .05$. B, Stained SDS-PAGE (upper panel) and Western blot (lower panel) of 0.2× SG of kDa marker (lane 1), GFP-dsRNA- (lane 2) and CFSH-dsRNA (lane 3) female and a single male (lane 4).

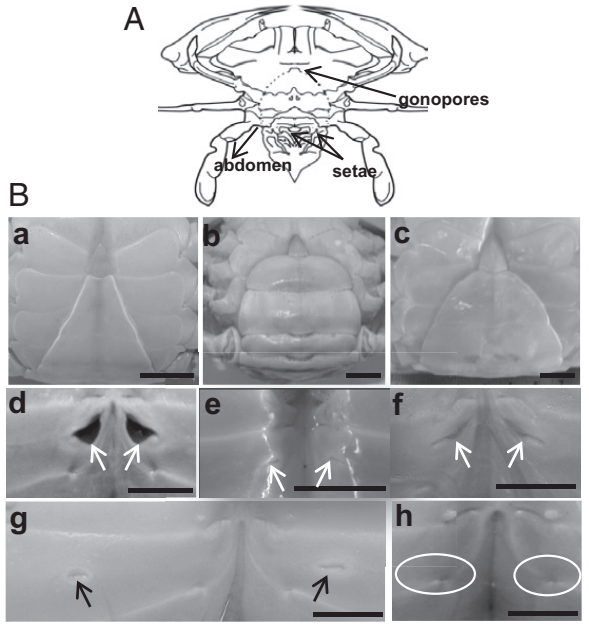

Figure 5. *CFSH*-knockdown impairs the development of the gonopores A, Schematic illustration (ventral view) of a normal mature female blue crab with closed or open abdominal cover. The internal side of the circular abdomen cover carries 5 pairs of pleopods with developed setae. Under the abdominal cover, a pair of gonopores flanks the center of the body. A dashed line marks the position of the abdominal cover when it is in its closed position. For more details see http://www.bluecrab.info/anatomy.html. B, a–c, The different shapes of the abdomen: a, triangular abdominal shape of a normal prepubertal female; c, semicircular abdominal shape of a normal adult female; b, dome shape abdomen of an eyestalk-ablated adult female; d–h, the gonopores revealed under the abdominal cover; d, a pair of large gonopores of a representative control *GFP-dsRNA* female vs smaller gonopores of an eyestalk-ablated (e) and a *CFSH-dsRNA*-injected females (f). The gonopores may also appear misplaced (g) or absent (h) in *CFSH-dsRNA*-injected females. The gonopores are marked with arrows or circled. Scale bar, 10 mm.

the plumose setae (Fig. 6E). In particular, *CFSH-dsRNA* treatments resulted in approximately 50% reduction in the length (12 ± 4 mm; n = 7). There were fewer ovigerous setae on the first and fourth pair of pleopods (Figure 6, D and F), than those of the *GFP-dsRNA*-treated females (25 ± 3 mm, n = 13; Figure 6, E and G).

Discussion

This study describes the presence of a female sex-dominant hormone playing integral roles in the development of brooding and mating systems that are displayed at the onset of puberty. In most mammalian and nonmammalian vertebrates, the endocrine function of the gonochoristic gonads plays a critical role in sex differentiation. However, although most malacostracan crustaceans are gonochoristic and display sexual dimorphism, sex differentiation is suggested to be governed extragonadally, primarily by the AG but also by several other endocrine organs including the MTXO, the molting glands -Y organs, and mandibular organs (28, 29).

Reproduction is controlled by specific hormones as neuronal and/or peripheral glandular controls to express timely behavior and physiology but the underlying mechanism is not known in all animal species. For the first time in crustaceans, a substance that is critically involved in the control of expression of relevant adult anatomic features in females is described. To date, insects, the most abundant and largest class in the animal kingdom, display considerable variation of parental care forms. However, only JH is associated with this process. JH stimulates gonadal development and negatively regulates parental care, eg, JH levels in the hemolymph inversely correlate with parental care (30). It is thus likely that JH alone cannot fully address the control of the diverse and sometimes complex reproductive strategies seen in this group. Thus, the discovery of a positive regulator of brooding and mating structures in crustaceans suggests that such a substance may also exist in insects.

Like other animals, crustacean females display morphologic structures specific to adults, which vary based on reproductive strategy, the degree of parental care, and the type of mating. Brachyuran (of the suborder Pleocyemata) females, including *C. sapidus* and *C. maenas* (31), form on their bodies external structures at the pubertal (or pubertal-terminal) molt in order to provide extended parental

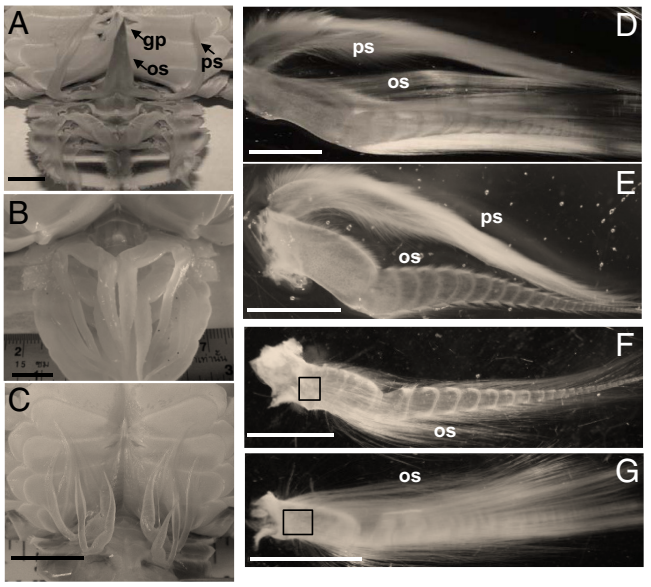

Figure 6. *CFSH* knockdown impairs the development of parental care system: the ovigerous setae A, Setae and pleopods located under the abdominal cover of a representative control *GFP-dsRNA*-treated female. B, Setae of an eyestalk-ablated females. C, Setae of *CFSH-dsRNA*-injected females. The first and fourth pair of pleopods of control *GFP-dsRNA* (D and G) and *CFSH-dsRNA* (E and F). Square marked is the area where the setae were measured for their length. Shorter and fewer ovigerous setae are notable in *CFSH-dsRNA*-treated animals. gp, gonopores; ps, plumose setae; os, ovigerous setae. Scale bar, 10 mm.

care for brooding embryos until hatching into viable larvae (32, 33). They also develop a pair of internal spermathecae to facilitate spermatophore storage for up to 2 years, ie, until the fertilization events that take place prior to extrusion of eggs (spawning) (34). Females belonging to the suborder Dendrobranchiata (such as prawns), known as egg releasers, do not develop such adult-specific systems because they do not need to provide extended parental care for their offspring or store spermatophores. Thus they may not have/need functional CFSH. The *CFSH* knockdown experiments clearly show that CFSH is required for the development of these 2 essential systems for successful mating and extended parental brood care in *C. sapidus* adult females.

The finding of a female-related substance was anticipated based on the observation that eyestalk-ablated prepubertal female crabs lack adult-specific phenotypes and, unlike intact females, continue to grow via molting (20, 21). This led to the identification of a peak containing CFSH in the SG neuropeptide RP-HPLC separation profiles of mature females, but not in those of males. The molecular weight of CFSH (~20 kDa), which is considerably higher than that of the CHH neuropeptide family (~8–9 kDa) (27, 35) excluded it as a potential member of this neuropeptide family. In fact, the deduced CFSH is a unique protein, with no homologuous sequences or conserved domains found in the GenBank (NCBI) and SwissProt. The predicted 23-aa signal peptide and 33-aa precursor-related peptide indicate that it is produced as a preprohormone-like CHH and as a secreted protein. This prediction is supported by the finding of immunoreactivity for CFSH in the MTXO, axonal tract, and the SG. Moreover, the N terminus sequence obtained by the Edman N-terminal sequencing confirms the cleavage site between the predicted end of the precursor-related peptide and the mature protein (Figure 2A). The remaining peaks, being the signature elution profile of SG neuropeptides found in several crab species (25, 36, 37) have been identified as CPRP, CHH-1 and -2, and MIH (25). These neuropeptides are not sex specific and are present in both sexes, with slightly higher quantities in males than females (as has also been reported in *C. maenas* (38)), indicating that CFSH is the only hormone present in the SG that shows a gender-specific difference in the levels.

Anti-CFSH serum specifically detects CFSH in distinct perikarya in the MTXO and in the SG of the eyestalk ganglia of adult females. Anti-CFSH serum specificity to CFSH is evident by the absence of staining with the anti-CFSH serum preincubated with rCFSH (Figure 3B′). Interestingly, a strikingly high intensity of CFSH immunostaining is found in the perikarya and along the axonal tract, which is in contrast to the weak staining in the SG that serves as the storage and release site of the CHH neuropeptides produced in the neurosecretory cells of the MTXO (27, 35, 39). This difference may indicate that, unlike CHH and MIH, which are being stored in large amounts in the SG (27, 35, 37, 38), CFSH is not stored and is probably being discharged from the SG immediately upon transport.

The ontogenic manifestation of a sex-related substance(s) regulating sexual dimorphism has never been described in crustaceans. The ontogenic presence of CFSH in the SG of *C. sapidus* occurs as early as at hatching-stage embryos. The observation that it is prevalent only in some embryos raises the possibility that early appearance of CFSH is linked to sex and perhaps found only in females. However, sex of the embryos was not determined and cannot yet be confirmed.

An endocrine interaction between the eyestalk ganglia and the androgenic gland has been suggested because eyestalk ablation of males results in hypertrophy of the androgenic gland and up-regulation of the AGH/IAG expression (43–45). However, the eyestalk-borne substance that regulates the activity of the androgenic gland has not yet been identified in any crustacean species (46). The exact profiles of *CFSH* and *AGH* expression throughout male and female life cycles need to be determined in order to better understand the functional significance of CFSH during development of the 2 sexes and whether, in fact, it is involved in early sex differentiation.

CFSH identified in the eyestalk ganglia of *C. maenas* shares high sequence identity (92%) with the *C. sapidus* CFSH (153/167 aa; Supplemental Figure 3). Despite the partial sequence, *CFSH* seems to be the most conserved neuropeptide found in the eyestalk ganglia to date. As a comparison, CHH and MIH of these 2 crab species show approximately 83% sequence identity (27), whereas decapod crustacean IAGs display very low (~25%) sequence identity (44). Such a high homology strongly suggests that CFSH has functions that are conserved in these 2 crab species. Moreover, because internal fertilization and the extended maternal brood care are not exclusive to brachyurans and can also be observed in other decapod crustaceans (34), CFSH may exist, with conserved functions, in other species that display similar reproductive strategies.

Gene silencing using dsRNA successfully yielded a specific knockdown of *CFSH* expression levels by 80%–90%, similar to the levels of males. The knockdown treatment was as effective in females as the *IAG-dsRNA* treatment was in males in a previous study in which the regeneration of masculine appendices was inhibited (10). The specificity of the treatment is confirmed as *MIH* transcript levels in the same eyestalks remain unchanged. Such reduced levels of *CFSH* were corroborated and reflected by the absence of the CFSH protein from the SG of *CFSH-dsRNA*-treated females. The similar results obtained from eyestalk-ablated and *CFSH*-knockdown treatments imply that the changes observed in the ovigerous setae and gonopores are intimately attributed to the reduced levels of *CFSH* expression. We have not observed a notable effect on molt increment or frequency, which reaffirms that CFSH may not be involved in the regulation of molting. Overall, the females treated with *CFSH-dsRNA* seem to have a reduced and compromised capacity for brooding and mating.

Sexual differentiation and dimorphism occurs gradually over several defined sequential stages during the life cycle of animals, including crustaceans (40, 42). The first significantly noticeable abnormality of the *CFSH* knockdown is observed in very young juvenile females (30–40 mm carapace width) displaying mispositioned gonopores. However, the most striking abnormality is seen in the pubertal terminally molted adult females exhibiting the poorly developed, ovigerous setae-carrying embryos. These observations suggest that CFSH is required continuously during juvenile stages, as well as at the pubertal molt, for the development of a reproductively competent adult female. On the other hand, the development of the spermathecae is not affected, suggesting that CFSH does not regulate all adult-stage-specific features. Additionally, it is unlikely that CFSH influences the onset of ovarian development, because the ovaries of females injected with *CFSH-dsRNA* develop similarly to those of the controls. At this point, however, we cannot rule out the possibility that a more dramatic decrease in CFSH levels or a complete knockout will negatively impact these features.

Our results provide the first definitive evidence for the presence of an endogenous female-specific hormone in arthropods: a hormone that positively regulates the development of adult stage-dependent female reproductive structures that are essential for the extended brooding and mating systems. We infer that CFSH facilitates both the development and the maintenance of the adult female reproductive features. Our findings indicate that the endocrine control and expression of specific reproductive strategies are extended to crustaceans, allowing broader comparative and evolutionary studies in the future.

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