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Molecular cloning and expression of caspase-3 in the protandrous cinnamon clownfish, *Amphiprion melanopus*, during sex change

Na Na Kim · Jehee Lee · Hamid R. Habibi · Cheol Young Choi

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Abstract The caspase-3 appears to be a key protease in the apoptotic pathway. We identified caspase-3 complementary DNAs from the ovaries of the protandrous cinnamon clownfish (*Amphiprion melanopus*), and investigated its mRNA and proteins, and activity levels during the sex change (I, mature male; II, male at 90 days after removing of the female; and III, mature female). The nucleotide sequence of the caspase-3 cDNA was 969 base pairs in length with open reading frames encoding peptides of 282 amino acids. The caspase-3 mRNA and protein, and activity levels in stages of the mature gonad are higher than those of the development gonad stage. To understand the effect of gonadotropin-releasing hormone (GnRH) on gonad apoptosis, we examined expression of genes caspase-3 mRNA and activity level in immature cinnamon clownfish gonads after GnRH analogue (GnRHa). The findings support the hypothesis that caspase-3 expression is associated with both testicular and ovarian development, and suggests that it may play a role in the control of ovarian development in cinnamon clownfish. Also, we demonstrate that GnRH agonists stimulate caspase-3 production which can in turn stimulate apoptosis. The present study provides a framework for better understanding of the role of caspase-3 during sex change processes in fish.

Keywords Apoptosis · Caspase-3 · Cinnamon clownfish · *Amphiprion melanopus* · GnRHa

N. N. Kim · C. Y. Choi (✉)
Division of Marine Environment and Bioscience, Korea Maritime University, Busan 606-791, Republic of Korea
e-mail: choic@hhu.ac.kr

J. Lee
Department of Marine Life Sciences, Jeju National University, Jeju Special Self-Governing Province, Cheju 690-756, Republic of Korea

H. R. Habibi
Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, AB, Canada

Introduction

Apoptosis is a form of cell death characterized by a number of biochemical and morphological changes, including DNA fragmentation, formation of apoptotic bodies, and a minimal inflammatory response (Kerr et al. 1972; Hacker 2000) in vertebrate. In most cases, the apoptotic process converges into the activation of caspases, a family of cysteine-dependent aspartate-specific proteases that are present in the cell as proenzymes (Earnshaw et al. 1999). Members of the caspase family share similarities in amino acid sequence and structure, and can be broadly divided into two groups: (1) those related to caspase-1 (caspases-1, 4, 5, 11, 12, 13, and 14), whose primary role appears to be in cytokine processing during inflammatory responses, and (2) apoptosis initiators or effectors (caspases-2, 3, 6, 7, 8, 9, and 10).

In particular, caspase-3 appears to be a key protease in the apoptotic pathway (Porter and Janicke 1999) and the most characterized effector caspase. Activated caspase-3 stimulates apoptosis by cleaving and inactivating a number of molecules, leading to cellular characteristics and morphological changes typically seen in apoptotic cells (Fischer et al. 2003). The resultant caspase-3 activates an endonuclease (caspase-activated deoxyribonuclease), which is partially responsible for the low-molecular-weight pattern of DNA fragmentation observed during apoptosis (Sakahira et al. 1998; Enari et al. 1998). In addition, caspase-3 levels were reported to increase in follicles undergoing atresia (Boone and Tsang 1998). Thus, caspase-3 seems to be involved in several cell death processes in the ovary.

Teleosts rely heavily on various environmental cues, such as temperature and light, during spawning (Billard 1986), and leads to gonadal regression, a process that may occur via the induction of apoptosis. In particular, apoptosis due to hormones and other chemicals that occurs during embryonic development and metamorphosis is involved in cell death (Corriero et al. 2009). Furthermore, testicular apoptosis is also known to occur during normal spermatogenesis in both mammals (Hong et al. 2008) and fish (Prisco et al. 2003), and it is thought to be essential for maintaining the correct ratio between sertoli cells and gametes during this process (Lee et al. 1997). During the gonadal development of juvenile zebrafish (*Danio rerio*), all animals initially contain undifferentiated ovary-like gonads, regardless of their genotypic sex (Takahashi 1977). Apoptosis mediates disappearance of oocytes within gonads as they change from ovary-like tissue to phenotypic testes in developing juvenile zebrafish (Uchida et al. 2002). There is evidence that gonadal apoptosis is mediated by both gonadotropins and locally produced hormones such as gonadal steroids (Billig et al. 1996) and peptides. In particular, gonadotropin-releasing hormone (GnRH) was shown to induce apoptosis in the testis of mature goldfish (*Carassius auratus*) (Andreu-Vieyra and Habibi 2001). GnRH, not a circulating hormone, plays a central role in the regulation of gonadal maturation and reproduction in fish and other species (Kavanough et al. 2008). In gonad of gilthead seabream (*Sparus aurata*), changes in the endogenous GnRH transcripts were shown to be associated with sex change and gonadal differentiation, and treatment with GnRH stimulate apoptosis (Soverchia et al. 2007). There is evidence that steroid hormones may protect ovary and testis against apoptosis. In this context, in rainbow trout (*Oncorhynchus mykiss*), apoptosis of preovulatory ovarian follicles can be suppressed by the administration of partially purified gonadotropin, estradiol-17β (E_2), or epidermal growth factor (Janz and Van Der Kraak 1997).

Social group of cinnamon clownfish (*Amphiprion melanopus*) typically consist of a mated adult pair and an immature individual, and the sexes of the fish are controlled by the social rank in the group. In general, the female is the largest of these fish and is dominant in the group. However, if a dominant female dies or is absent, the male partner undergoes a sex change to female (Godwin and Thomas 1993; Godwin 1994). Hence, cinnamon clownfish is a good model for studying the mechanism of sex change in protandrous hermaphroditic fish. To date, researchers have investigated the changes in steroid hormones (Godwin and Thomas 1993), the expression of maturation or differentiation genes (Kim et al. 2010), and the changes in gonads histology (An et al. 2010) that occur during sex change processes in cinnamon clownfish, but no studies have been reported on apoptosis involved in these processes.

To investigate the roles of caspase-3 in cinnamon clownfish sex change processes, we isolated complementary DNAs (cDNAs) of caspase-3 from cinnamon clownfish and studied its mRNA level, activity, and proteins expression during sex change process. In addition, we investigated the effects of GnRH analogue (GnRHa) on caspase-3 mRNA expression and activity in immature cinnamon clownfish.

Materials and methods

Experimental fish

This study was conducted on different subtypes of cinnamon clownfish, including mature males (I, 10.5 ± 1.2 g), males 90 days after removing females (II, 15.2 ± 0.9 g), and mature females (III, 22.2 ± 2.1 g). Fish were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea). Each group consisted of a mated pair (dominant female and male). Moreover, each group was reared in one of fifteen 50-l circulation filter tanks prior to inclusion in the experiments. The water

temperature was maintained at 28 ± 0.5 °C, and the photoperiod was a 12:12-h light–dark cycle. Fish were fed a commercial marine aquarium fish feed (Jeilfeed Co., Kyoungnam, Korea) twice a day.

The experimental design used for sex change in the present study was modified from the methods of An et al. (2010). Briefly, after random assignment of the groups, each consisting of a mated pair with a dominant female and a male, sex change was induced in each male fish by removing the paired female from each group and adding an immature clownfish for pairing with the male in experimental condition. We observed the male fish undergoing the sex change process from male to female at 90 days after removing the paired females. We divided the process of sex change into three developmental stages as follows: (I) mature male; (II) male at 90 days after removing female; and (III) mature female. The fish were anesthetized in tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) and decapitated prior to tissue collection. Gonads from the fish at each gonad maturity stage were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis.

cDNA cloning of caspase-3

Partial fragments of the caspase-3 gene were amplified as follows: Primers for caspase-3 were designed using highly conserved regions of European seabass caspase-3 (*Dicentrarchus labrax*, Accession no. DQ345773), large yellow croaker caspase-3 (*Larimichthys crocea*, Accession no. EU878546), pufferfish caspase-3 (*Takifugu rubripes*, Accession no. NM001032699), and Atlantic salmon caspase-3 (*Salmo salar*, Accession no. BT059710), leading to caspase-3 forward primer (5′-CGA CCA GAC AGT GGA GCA AAT G-3′) and caspase-3 reverse primer (5′-AGA TCG GTG CCT CTG CAA G-3′).

Total RNA was extracted from the gonad by using the TRIzol kit (Gibco/BRL, Gaithersburg, MD, USA), which is free of DNA contamination. Reverse transcription (RT) was conducted using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea), and polymerase chain reaction (PCR) amplification was performed using 2× Taq Premix I (Solgent, Seoul, Korea) according to the manufacturer's instructions. RT was carried out at 42 °C for 50 min. PCR was subsequently performed for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 2 min, denaturation at 94 °C for 20 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1 min, followed by 1 cycle of final extension at 72 °C for 7 min. Amplified PCR products were processed by electrophoresis using a 1 % agarose gel containing ethidium bromide. The PCR products were purified and subsequently cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). The colony formed by transformation was cultivated in DH5α cells (RBC Life Sciences, Seoul, Korea). The plasmid DNA was subsequently extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo Genetech, Seoul, Korea), and the insert DNA was separated using EcoRI (Fermentas, Glen Burnie, MD, USA). Based on the plasmid DNA, caspase-3 cDNA sequences were analyzed using an ABI DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA).

Rapid amplification of caspase-3 cDNA 3′ and 5′ ends (3′ and 5′ RACE)

Total RNA was extracted from gonads using a TRIzol kit (Gibco/BRL, NY, NY, USA) 3 μg of total RNA as a template, and 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was conducted using an oligo-$d(T)_{18}$ anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene).

Caspase-3-specific primers were selected from the PCR product obtained from RT-PCR. For 3′ RACE, the 50 μl PCR mixture contained 5 μl of 3′ RACE cDNA, 1 μl of 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μl of 10 mM 3′ RACE caspase-3-specific primer (5′-ATG ACT CCT GCG ATT ACA CGT GTC GTC-3′), and 25 μl of SeeAmp Taq Plus master mix (Seegene). PCR was performed for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by 1 cycle of final extension at 72 °C for 5 min.

For 5′ RACE, the 50 μl PCR mixture contained 5 μl of 5′ RACE cDNA, 1 μl of 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μl of 10 mM 5′ RACE caspase-3-specific primer (5′-TCA TGG TGT TCC TCC ACG

AGT AGT AGC-3′), and 25 μl of SeeAmp Taq Plus master mix (Seegene). PCR was performed using the protocol described above for 3′ RACE.

Amplified PCR products were processed by electrophoresis using 1 % agarose gels containing ethidium bromide. The transformation and sequencing were performed using the same method as described above in cDNA cloning of caspase-3 section.

Sequence data analyses of caspase-3

The predicted amino acid sequences of caspase-3 in cinnamon clownfish were deduced using Expasy Translate Tool (http://www.expasy.org) and that was modified from the methods of Monteiro et al. (2009). The nucleotide and protein full sequences were compared to sequences available in the GenBank database retrieved using the BLAST program (http://www.ncbi.nlm.nih.gov/), and multiple alignment were made using the CLUSTAL W program (www.ebi.ac.uk/clustalw/index.html). Caspase-3 domains and possible N-glycosylation sites were based on PROSITE predictions (www.expasy.org/prosite). The percentages of similarity and identity were calculated by pair-wise alignments by the program needle at www.ebi.ac.uk, with first and extending gap of 10 and 0.5, respectively.

Phylogenetic analysis of caspase-3

Caspase-3 phylogenetic analysis was conducted using known vertebrate caspase-3 amino acid sequences aligned using BioEdit software (Hall 1999). The phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

Production of caspase-3 polyclonal antibody

To obtain the antigen of the cinnamon clownfish caspase-3 antibody, a synthetic peptide was synthesized at Cosmo Genetech (Seoul, Korea) and was coupled to bovine serum albumin (BSA). The peptide chain was designed against highly conserved regions of caspase-3 amino acid sequences of cinnamon clownfish and other teleosts and caspase family (Fig. 1; 182–198; CGIETDSGEDTTKIPVE-C). A rabbit was injected with 100 μg of BSA-conjugated synthetic peptide in Freund's complete adjuvant and boosted at 2-week intervals by subcutaneous injection of 200 μg of BSA-conjugated synthetic peptide in Freund's incomplete adjuvant. The rabbit was bled 1 week after the fifth injection. Antiserum was purified with affinity peptide column coupling using the same peptide as described above (Lu et al. 2000). This antibody recognizes full-length cinnamon clownfish caspase-3 that obtain bands in the 32 kDa. The titer and specificity of the purified antibody were tested with ELISA and Western blotting analyses.

Direct ELISA assay of antibody

To demonstrate the specificity of cinnamon clownfish caspase-3 antibody, it was preincubated with purified antibody. The activity of the isolated antibody was measured by an indirect ELISA test (Magnadottir et al. 1999). We have direct ELISA test, on 3 times boosting (2, 4, and 6 weeks) and 2 times serum bleeding (3 and 5 weeks). We applied capture antibody titer by adding antigen caspase-3-peptide to the appropriate wells (500 ng/well) of microtiter plates (96-well Nunc Maxisorp; Nunc, Roskilde, Denmark). Microtiter plates adding antigen caspase-3-peptide and coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$; 500 ng/well).

The plate was incubated at 4 °C overnight, subsequently washed three times with PBST [containing 0.05 % Tween-20 in phosphate buffered saline (PBS)], and well dried for 37 °C 30 min. 200 μl of blocking solution was added to 5 % skim milk in PBST in each well, and the plate was incubated at room temperature for 1 h. The plate was subsequently emptied and washed with PBST. Detection antibody was applied by adding caspase-3 peptide antibodies to appropriate wells (1 μg/ml, 100 μl/well). The microtiter plate was again incubated at room temperature for 2 h. Subsequently, the plate was emptied and washed three times with PBST. The secondary antibody was applied by adding horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) to appropriate wells. The plates were sealed and incubated at room temperature for 1 h. The plates were washed five times with PBST, and the peroxidase activity was revealed by adding 50 μl of substance D (*o*-Phenylenediamine dihydrochloride [OPD], 400 μg/ml, 0.03 % H_2O_2, citrate buffer). The enzyme reaction was stopped after 30 min by addition of 100 μl solution (1 N H_2SO_4), and the absorbance was read at 450 nm using a microplate reader.

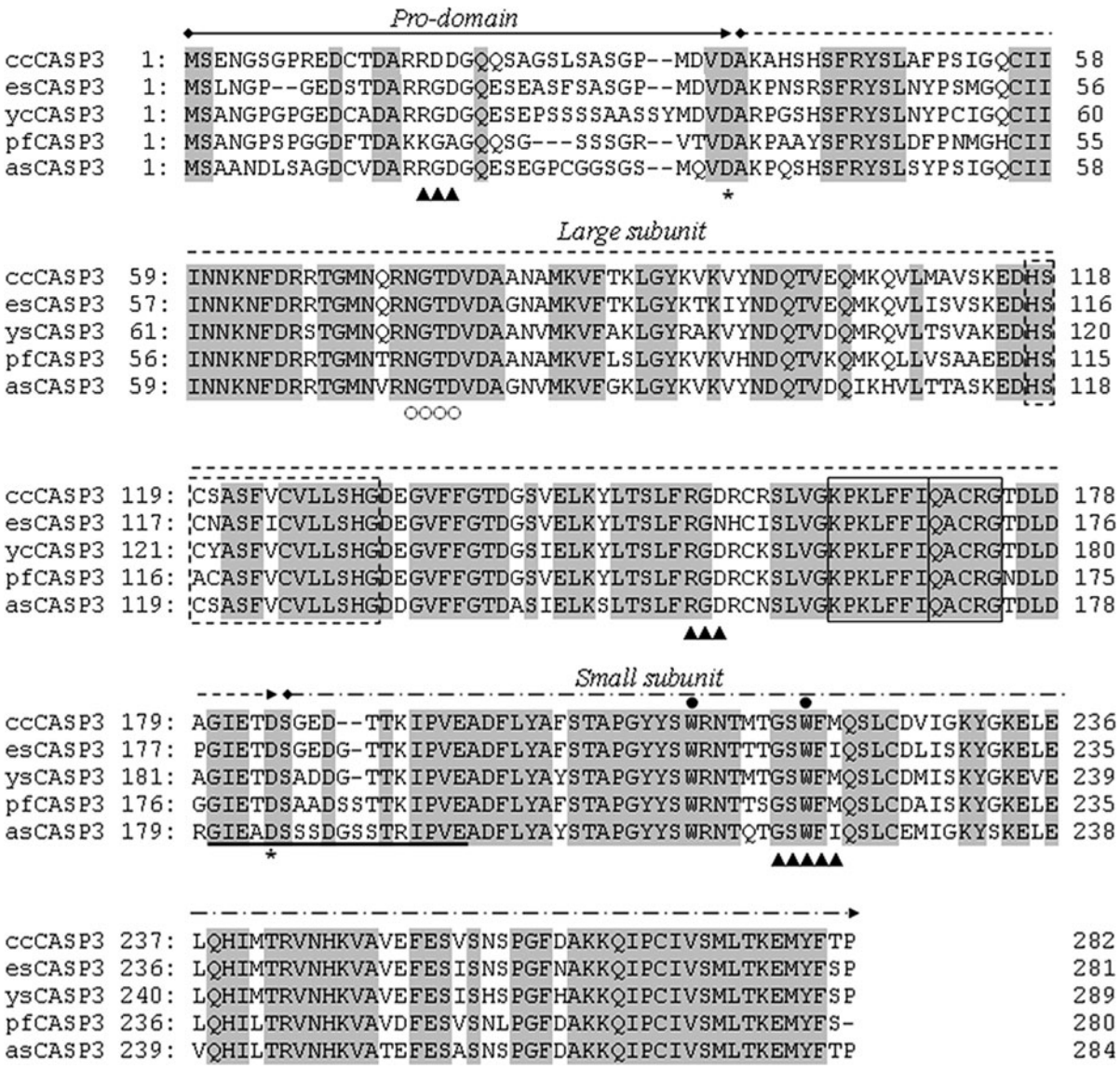

Fig. 1 Alignment of the amino acid sequences of caspase-3 from the cinnamon clownfish (ccCASP3, JF345174), European seabass (*Dicentrarchus labrax*; Accession no. esCASP3, DQ345773), yellow croaker (*Larimichthys crocea*; ycCASP3, Accession no. EU878546), pufferfish (*Takifugu rubripes*; pfCASP3, Accession no. NM001032699), and Atlantic salmon (*Salmo salar*; asCASP3, Accession no. BT059710). The *shades* indicate amino acids that are identical to those in the first line, which correspond to the ccCASP3. The *dashes* indicate gaps inserted to enhance sequence similarity. The pentapeptide active-site motif (QACRG) and the caspase family signature are *boxed* in *continuous* and *discontinuous lines*, respectively. The cysteine active site of the caspase family is *boxed* in a *continuous line,* and N-glycosylation site (NGTD) is in *open circles*. The protein-binding domain (GSWFM) and RGD motifs are in *filled triangles*. The putative cleavage site at the aspartic acid residues, which separates the pro-domain (*right arrow*) from the large subunit (*right arrow*) and from the small subunit (*right arrow*), is indicated with *asterisks*. The two tryptophan residues that undergo rearrangement when caspase-3 is activated are also indicated with *filled circles*. The synthesized peptide sequence for the antigen of the cinnamon clownfish caspase-3 antibody is in *bold line* in caspase-3 (180–195). The *numbers* indicate the amino acid positions, and the *dashes* indicate the gaps introduced to optimize similarity between sequences

Western blot analysis

Total protein isolated from the gonads during the sex changes in cinnamon clownfish was extracted using protein extraction buffer [5.6 mM Tris, 0.55 mM ethylenediaminetetraacetic acid (EDTA), 0.1 % sodium dodecyl sulfate (SDS), 0.15 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 0.15 mg/ml leupeptin], sonicated, and quantified using the Bradford method (Bio-Rad). Total protein (30 μg) was loaded per lane onto a 4 % acrylamide stacking gel and a 12 % acrylamide resolving gel. For reference, a protein ladder (Fermentas) was also used. Samples were electrophoresed at 80 V until they moved through the stacking gel and at 150 V while they moved through the resolving gel, until the bromophenol blue dye front had run off the gel. The gels were immediately transferred to a 0.2 μm polyvinylidene diflouride (PVDF) membrane (Bio-Rad) and run at 85 V for 1.5 h at 4 °C. Thereafter, the membranes were blocked with 5 % milk in Tris-buffered saline (TBS; pH 7.4) for 45 min, and were subsequently washed in TBS. The membranes were incubated with polyclonal rabbit antibody to caspase-3 (dilution 1:1,000); an internal control was β-tubulin (ab6046, abcam, UK). The blots were further treated with a HRP-conjugated anti-rabbit IgG secondary antibody (dilution 1:5,000, Bio-Rad) for 60 min. Protein bands were detected using both the standard ECL system and the more sensitive ECL system (ECL Advance; GE Life Sciences, Uppsala, Sweden); exposure to autoradiography-sensitive film was performed for 5 min.

GnRH treatment

To investigate the roles of caspase-3 in the reproductive physiology of cinnamon clownfish, we treated immature male fish with GnRHa, and subsequently measured gonadal expression and activity of caspase-3. The fish were reared in 220-l circulation filter tanks in the laboratory, and were anesthetized with MS-222 (200 mg/l) prior to injection. GnRHa (des Gly^{10}-[D-Ala^{6}] LHRH ethylamide, Sigma) was dissolved in physiological saline (0.9 % NaCl). Each fish was given an intraperitoneal injection of GnRHa [0.1 and 0.5 μg/g of fish body weight (BW)] at a volume of 10 μl/g BW, and sham group of fish was injected with a dissolved equal volume of 0.9 % NaCl (10 μl/g BW). After injection, gonad samples were removed from five fish at each of the following time periods: 0, 6, 12, 24, and 48 h. During the experimental period, the water temperature and photoperiod were maintained at 28 ± 1 °C and 12:12-h light–dark ratio, respectively.

Quantitative PCR (QPCR)

QPCR was performed to determine the relative expression of caspase-3 mRNA in the gonads of cinnamon clownfish. Primers for QPCR were designed with reference to the known sequences of cinnamon clownfish as follows: caspase-3 forward primer (5′-CGA CCA GAC AGT GGA GCA AAT G-3′), caspase-3 reverse primer (5′-TAC CGA AGA ACA CGC CCT CAT C-3′), β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG -3′), β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′), GAPDH forward primer (5′-AGA AGG CCT CTG CTC ACT TG -3′), and GAPDH reverse primer (5′-TTG CTG ACA ACT GGG AGG GA-3′). PCR amplification was carried out using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System and iQ^{TM} SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was carried out as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s. After the QPCR, the data from three replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. As an internal control, experiments were duplicated with β-actin and GAPDH; all data were expressed as change with respect to the corresponding β-actin and GAPDH calculated cycle threshold (ΔCt) levels. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR, QPCR data from three replicate samples were analyzed with the software of the cycler system to estimate the transcript copy numbers for each sample.

Histological analysis

The gonad tissues from each developmental group (mature male, male at 90 days after removing female, and mature female) for the analysis of gonads during sex change were fixed in Bouin's solution. The samples were dehydrated in increasing ethanol

concentrations, clarified in xylene, and embedded in paraffin. Sections (5 μm thick) were selected and stained with haematoxylin–eosin for observation under a light microscope (Leica DM 100, Germany), and images were captured with a digital camera (Leica DFC 290, Germany).

Caspase-3 activity assay

Caspase-3 activity of gonads was measured using a commercially available kit (Sigma), according to the manufacturer's protocol. Cinnamon clownfish gonads were lysed in lysis buffer after treatment with hexane fraction (HF) (25, 50, 100, or 200 μg/ml) for 24 h. Active caspase-3 was used as a positive control. All mixtures were incubated overnight in a humidified environment at 37 °C. The amount of *p*-Nitroaniline (*p*-NA) released from the substrate was measured at 405 nm using a plate reader.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Tukey's post hoc tests was used to compare the differences in the data ($P < 0.05$). All values are expressed as mean ± standard error (SE).

Results

Characterization of caspase-3 sequence

The sequences of cinnamon clownfish caspase-3 cDNA (Accession no. JF345174) were obtained with RT-PCR using mRNA extracted from fish gonads (Fig. 1). For caspase-3, the cDNA sequence of 849 bp showed signif icant similarity with the caspase-3 of other vertebrates, as revealed by the BLAST results (www.ncbi.nlm.nih.gov). Analysis in the PROSITE database predicted that the cinnamon clownfish caspase-3 fragment contained caspase family p20 (S^{52} to D^{176}) and p10 (G^{180} to P^{282}) domain profiles, a caspase family histidine active site (caspase family signature; H^{117}SCSASFVCVLLSHG), and a caspase family cysteine active site (K^{163}PKLFFI-QACRG). An N-glycosylation site (N^{74}GTD) was also predicted. Furthermore, the pentapeptide active-site motif (Q^{168}ACRG) and the putative aspartic acid (Asp^{155}), which splits the large and small subunits, were conserved among all the sequences from the different species that were analyzed, as were the two subunits. The protein-binding domain GSWFM was also well conserved among the species analyzed, revealing only a conservative substitution in the last amino acid of the motif for Atlantic salmon. The two tryptophan residues that were previously shown to undergo rearrangement during activation of caspase-3, resulting in the reorganization of the active site (Park et al. 2004), were also conserved among the analyzed species, which suggests that the same process of reorganization can occur in fish. Furthermore, we found that an integrin-recognition motif (RGD) near the active site was conserved in all analyzed species. The cinnamon clownfish caspase-3 sequence exhibited a very similar homology to these of other fish: 85 % with European seabass, 85 % with large yellow croaker, 84 % with pufferfish, and 80 % with Atlantic salmon.

Phylogenetic analysis

To reveal the molecular phylogenetic position of cinnamon clownfish caspase-3, a phylogenetic tree was constructed. As shown in Fig. 2, caspase-3 is clearly separated into two clusters in vertebrates. Phylogenetic analysis showed that the cinnamon clownfish caspase-3 was closely related to caspase-3 of other teleost. The teleost caspase-3 was grouped together, respectively.

Direct ELISA assay for antibody characterization

On Western blot analysis, a new polyclonal antibody to caspase-3 produced in this study recognized a band with a molecular mass of 32 kDa in the cinnamon clownfish ovary (Fig. 4a). We have produced 32 kDa caspase-3 antibody, as the titer and specificity of the purified caspase-3 antibody were tested with ELISA. The ELISA method developed in this work is based on the use of antibodies against a synthetic peptide from the cinnamon clownfish sequence. Prior to ELISA development and validation, we assessed the specificity of anti-caspase-3 antibodies. The antibodies were characterized by direct ELISA analysis using the caspase-3 peptide (Fig. 3). These results indicated that anti-caspase-3 antibodies cross-reacted with caspase-3 peptide (2, 4, and 6 weeks), and exhibited a decrease in the signal proportional to the dilution of the anti-caspase-3 antibodies in the range of 1:1,000, 1:5,000,

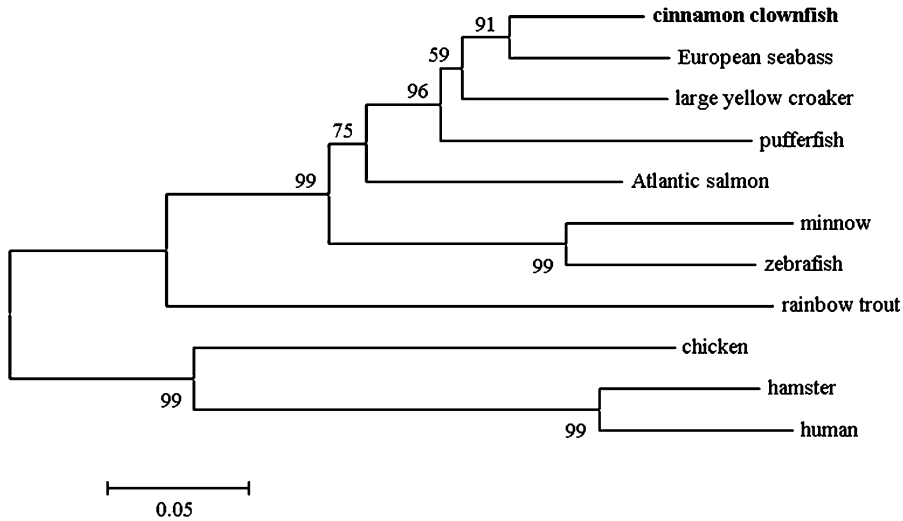

Fig. 2 Phylogenetic tree based on an amino acid alignment for caspase-3 in teleost fish. Bootstrap values (%) are indicated for 1,000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are as follows: caspase-3 cinnamon clownfish (Accession no. JF345174), European seabass (Accession no. DQ345773), large yellow croaker (Accession no. EU878546), pufferfish (Accession no. NM001032699), Atlantic salmon (Accession no. BT059710), minnow (Accession no. GQ406344), zebrafish (Accession no. AB047003), rainbow trout (Accession no. FR751081), chicken (Accession no. NM204725), Chinese hamster (Accession no. FJ940732), and human (Accession no. AJ413269)

and 1:10,000. Competitive ELISA development demonstrated the availability of a standard and an antibody specific of the protein.

Expression and activity of caspase-3 during the sex change process

The caspase-3 mRNA and protein expression were detectable in gonads from developmental stages of the sex change (Fig. 4). The expression of caspase-3 was investigated in the gonads by means of Western blot analysis, which revealed a protein with caspase-3 immunoreactivity of a size corresponding to the predicted size for cinnamon clownfish caspase-3 (32 kDa). The expression of this protein was correlated with the caspase-3 mRNA levels in cinnamon clownfish gonads. The overall results demonstrate that the caspase-3 activity is higher in mature gonads, compared to the development gonad stage.

Histological analysis

The testis of mature male mainly consisted of testicular tissue, with oogonia and primary oocytes (Fig. 5a). And then the oocytes had developed and increased in size (Fig. 5b), the diameter of oocytes was increased, the testicular tissue was regressed, and finally the fish became females (Fig. 5c).

Time-related effect of GnRHa on the expression and activity of caspase-3

Treatment with 0.1 and 0.5 μg/g of GnRHa significantly increased caspase-3 mRNA level in cinnamon clownfish gonads (Fig. 6a). The GnRHa-induced caspase-3 mRNA was approximately 79.3 and 78.1 times higher (than the untreated control level) at 6 h (at 0.5 μg/g GnRHa) and 12 h (at 0.1 μg/g GnRHa), respectively. The activity of caspase-3 mRNA was maintained at 5.5 times more than the control level following injection with GnRHa (Fig. 6b).

Discussion

We isolated full-length caspase-3 from the gonads of cinnamon clownfish, and investigated the changes in mRNA level, activity, and histological during the sex change from male to female. We further investigated the effect of GnRHa on caspase-3 mRNA level and activity in immature cinnamon clownfish.

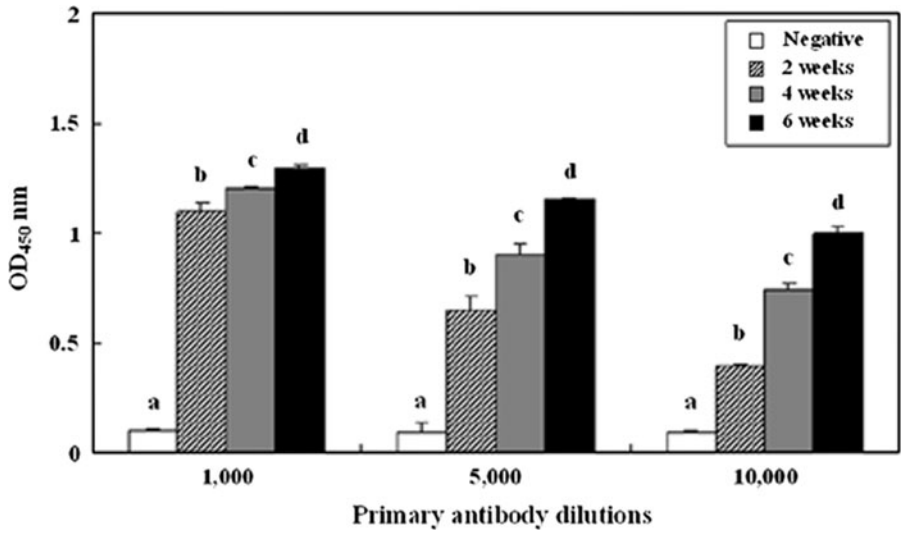

Fig. 3 Direct ELISA was performed with induced (2, 4, and 6 weeks) and non-induced (negative) samples from different fish species diluted to 1:5,000 and various dilutions (1,000, 5,000, and 10,000) of caspase-3 antibodies

Caspase-3 cDNA contained 969 nucleotides, including an open reading frame predicted to encode a protein of 282 amino acids (Fig. 1). The results provide information on caspase family p20 and p10 domain profiles, and demonstrate that caspase family signature and cysteine active site are well conserved in the caspase-3 sequence. The pentapeptide active-site motif QACRG was also conserved and interrupted after its first amino acid (Q) by an intron, as in all species analyzed herein. It has been established previously that the active form of caspase-3 is generated after proteolytic cleavage of the proenzyme at aspartic acid residues (Wang and Keiser 1998). In the presented alignment (Fig. 1), the cleavage sites of caspase-3, predicted at Asp^{36} (D^{36}) and Asp^{184} (D^{184}), separates the prodomain, large subunit, small subunits, and an RGD upstream of the active site. These sequences are conserved in all species analyzed except for the cinnamon clownfish sequence in which the aspartate residue is replaced by an aspartic acid (D^{155}). Aspartate-aspartate-X (DDX), located near the site of processing ($D^{28}DG$) that produces the p12 and p17 subunits, interacts with RGD peptides, leading to direct triggering of conformational changes that promote pro-caspase-3 autoprocessing and activation (Pasqualini et al. 1995).

In this study, caspase-3 mRNA expression level was low in the male fish during the sex change, from male to female, after the removal of the paired female (Fig. 4). Also, caspase-3 mRNA expression decrease during stage II, in which the developing ovarian tissue did not show activations of caspase-3. However, the expression level increased in fish during change from male to female. The caspase-3 mRNA level was highest in the mature female. This was correlated with the observed increase in the activity of caspase-3 during the male-to-female sex change. The present study provides evidence that caspase-3 activity may be related to the sex change in clownfish. It can logically be postulated that caspase-3 may play a role in induction of testicular apoptosis in a process leading to the development of ovary and development of oocytes in protandrous cinnamon clownfish. Also, in Western blot analysis, the caspase-3 protein was similar to the mRNA expression and activity of caspase-3. These results are consistent with previous studies that demonstrated apoptosis during normal spermatogenesis and postovulatory regression in teleost gonad in rainbow trout (Uchida et al. 2002) and goldfish (Wood and Van Der Kraak 2001). Thus, the present results provide a support for the hypothesis that caspase-3 is involved in the development and maturity of gonads by stimulating apoptosis during the sex change from male to female in cinnamon clownfish. In zebrafish development, oocyte apoptosis induces testicular and ovarian differentiation (Uchida et al. 2002). In such oocytes, a proapoptotic signaling pathway specific to males may be present and it may be stimulated by sex

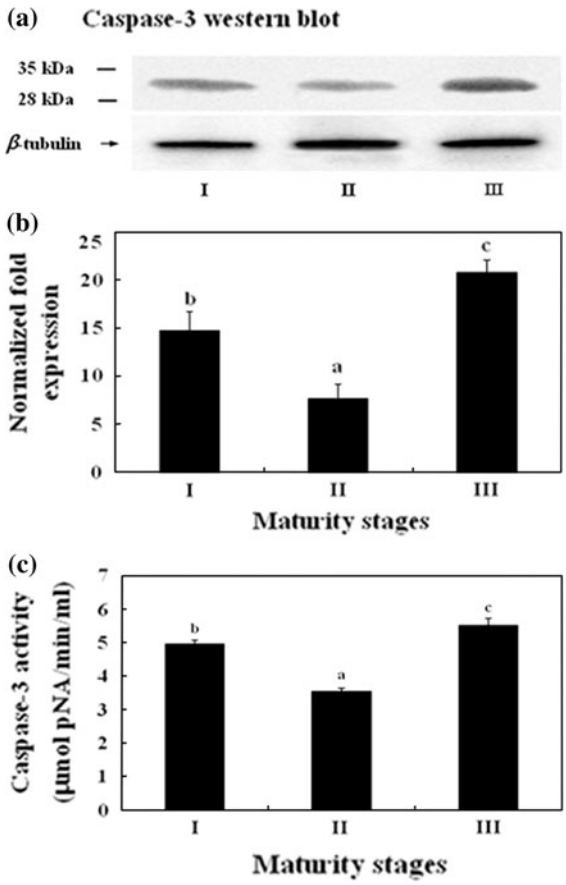

Fig. 4 Expression and activity of caspase-3 in gonads of cinnamon clownfish during the sex change (*I,* mature male; *II,* male at 90 days after removing female; *III,* mature female). **a** Western blot of caspase-3 protein expression in gonads of cinnamon clownfish during the sex change, and β-tubulin (55 kDa) was the internal control. **b** Caspase-3 mRNA levels relative to β-actin and GAPDH mRNA levels in the gonads of cinnamon clownfish during sex changes were analyzed with real-time PCR. **c** The activity of caspase-3 in the gonads of cinnamon clownfish during sex changes was also analyzed with a plate reader. Values with *dissimilar letters* are significantly different ($P < 0.05$) from each other. Values are expressed as mean ± SE ($n = 5$)

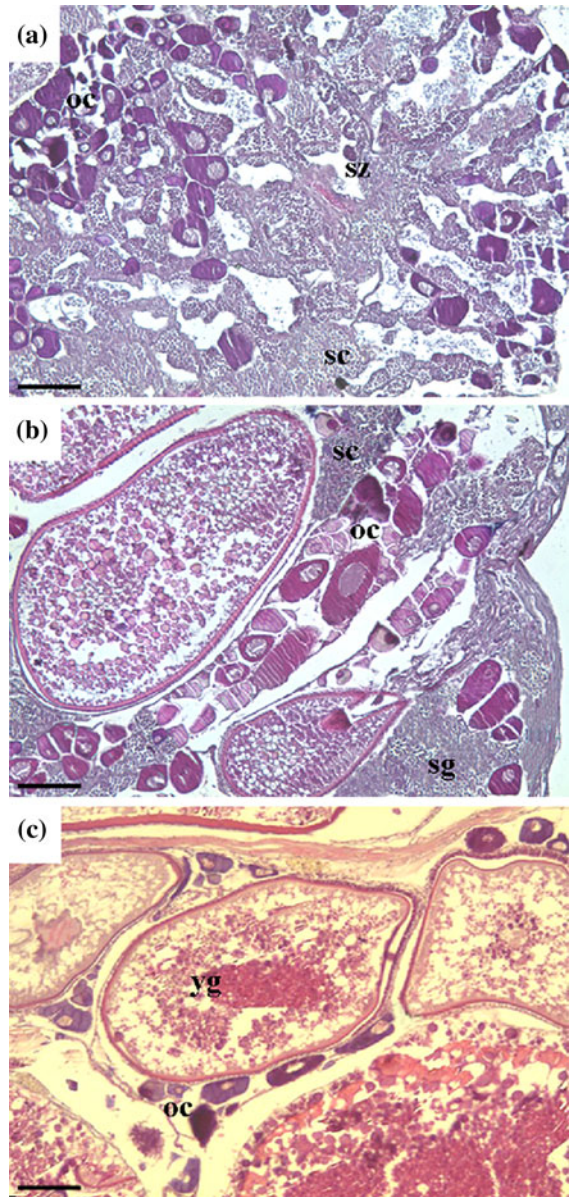

Fig. 5 Photomicrograph of gonad maturity stages during sex change in cinnamon clownfish. **a** Mature male (stage I in Fig. 4), **b** male at 90 days after removing female (stage II in Fig. 4), **c** mature female (stage III in Fig. 4). *st* spermatides, *sc* spermatocytes, *sg* spermatogonia, *oc* oocytes, *yg* yolk granules. *Scale bar* 100 μm. Reprinted from ref. (An et al. 2010), with permission from Comparative Biochemistry and Physiology, Part-A

determining genes or changes in estrogen or thyroid hormone levels. In addition, we examined gonadal histology during sex change in cinnamon clownfish. As a result, we found that the oocytes were developed and testicular tissue was regressed in the ovary as the cinnamon clownfish were changed from male to female (Fig. 5). This result was similar to the previous study (Godwin 1994). We therefore suggest that gonad development and maturation were controlled by the pituitary-gonad axis through increases in the levels of caspase-3 mRNA in cinnamon clownfish.

The present study taken further steps to investigate the role of GnRH in regulation of apoptosis in cinnamon clownfish. Injection with GnRHa significantly

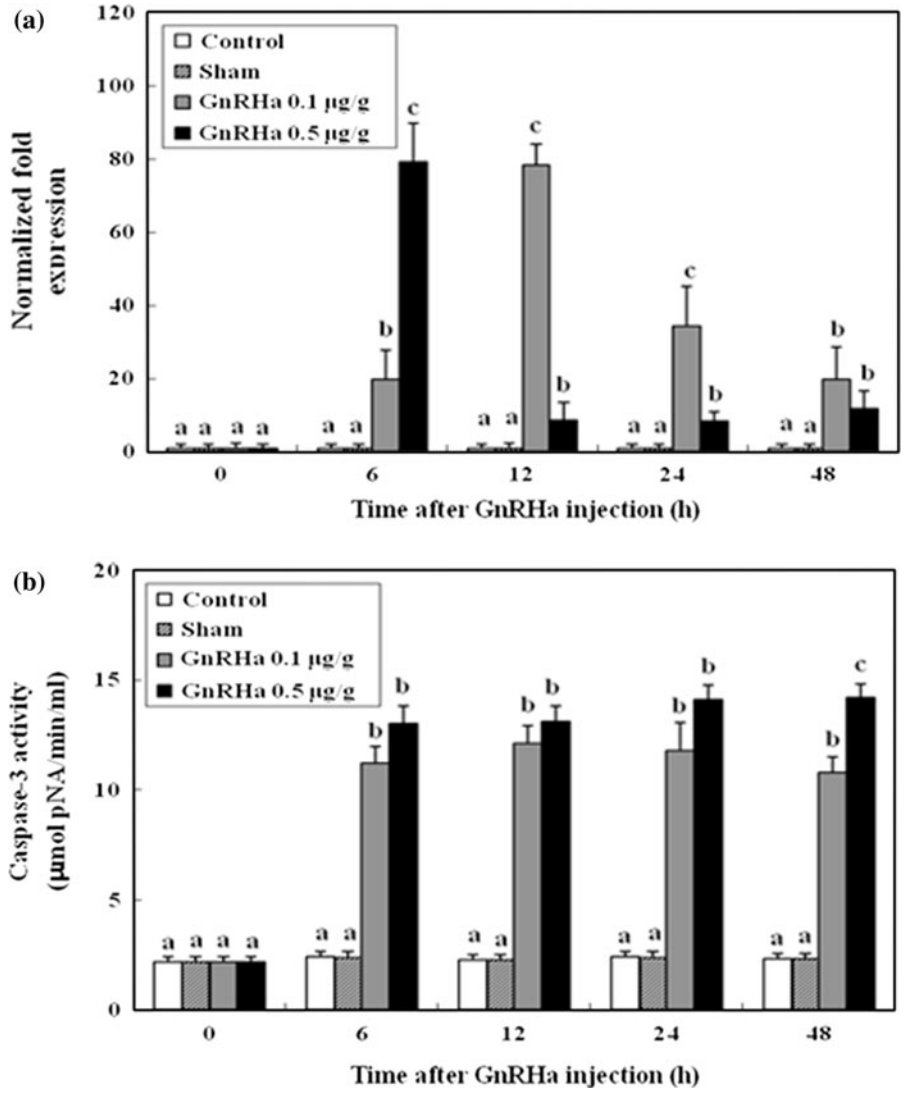

Fig. 6 Time-related effects of GnRHa (control, sham, 0.1, and 0.5 μg/g) on caspase-3 mRNA levels (**a**) and activity (**b**) in cinnamon clownfish gonads. Total RNA was extracted from gonads 0, 6, 12, 24, and 48 h after GnRHa treatment, and 3 μg was used for PCR. The expression level of each sample was normalized with respect to the *β*-actin and GAPDH signal, and is expressed as the relative expression level. Values with *dissimilar letters* are significantly different ($P < 0.05$) from each other. Values are expressed as mean ± SE ($n = 5$)

increased caspase-3 mRNA expression in immature cinnamon clownfish gonad (Fig. 6). The observed GnRH-induced increase in caspase-3 mRNA is consistent with previous report in goldfish (Andreu-Vieyra and Habibi 2001) and gilthead seabream (Soverchia et al. 2007). The stimulation of caspase-3 was observed at the highest dose of GnRHa tested presumably due to increasing proapoptotic proteins, opposing the antiapoptotic actions of gonadotropin hormones and growth factors (Parborell et al. 2002). Also, Andreu-Vieyra and Habibi (2001) was reported that the observed effect of GnRH on testicular apoptosis is significant, because local factors such as GnRH peptides are likely to play an important regulatory role in controlling testicular spermatogenesis during various reproductive cycles in goldfish. Recent studies have also suggested that GnRH induction of apoptosis correlates with an increased expression of the proapoptotic protein Bax, as well as with decreased basal testosterone production (Andreu-Vieyra et al. 2005). In addition, published studies have demonstrated that GnRH-induced apoptosis occurred only during the late stage of spermatogenesis (Chowdhury et al. 2008), and was mediated steroid hormone production as basal of testosterone levels (Soverchia et al. 2007). The present results confirm previous observations that GnRH plays an important role in the control of gonadal development by controlling apoptotic pathways in the testis and ovary (Andreu-Vieyra et al. 2005; Soverchia et al. 2007). Furthermore, we demonstrate an increase in caspase-3 production during

the male-to-female sex change process in clownfish. We also provide evidence that GnRH agonists stimulate caspase-3 production which can in turn stimulate apoptosis. The findings facilitate better understanding of the mechanisms underlying sex change in cinnamon clownfish and other hermaphrodite teleost fish.

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