

Phylogenetic analysis of the caspase family in bivalves: implications for programmed cell death, immune response and development

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Abstract

Background: Apoptosis is an important process for an organism's innate immune system to respond to pathogens, while also allowing for cell differentiation and other essential life functions. Caspases are one of the key protease enzymes involved in the apoptotic process, however there is currently a very limited understanding of bivalve caspase diversity and functions.

Results: In this work, we investigated the presence of caspase homologs using a combination of bioinformatics and phylogenetic analyses. We blasted the *Crassostrea gigas* genome for caspase homologs and identified thirty-five potential homologs in the addition to the already cloned twenty-three bivalve caspases. As such, we present information about the phylogenetic relationship of all identified bivalve caspases in relation to their homology to well-established vertebrate and invertebrate caspases. Our results reveal unexpected novelty and complexity in the bivalve caspase family. Notably, we were unable to identify direct homologues to the initiator caspase-9, a key-caspase in the vertebrate apoptotic pathway, inflammatory caspases (caspase-1,-4 or -5) or executioner caspases-3, -6, -7. We also explored the fact that bivalves appear to possess several unique homologs to the initiator caspase groups -2 and -8. Large expansions of caspase-3 like homologues (caspase-3A-C), caspase-3/7 group and caspase-3/7-like homologues were also identified, suggesting unusual roles of caspases with direct implications for our understanding of immune response in relation to common bivalve diseases. Furthermore, we assessed the gene expression of two initiator (Cg2A, Cg8B) and four executioner caspases (Cg3A, Cg3B, Cg3C, Cg3/7) in *C. gigas* late-larval development and during metamorphosis, indicating that caspase expressions vary across the different developmental stages.

Conclusion: Our analysis provides the first overview of caspases across different bivalve species with essential new insight into caspase diversity, knowledge that can be used for further investigations into response to pathogens or regulation of developmental processes.

Background

Bivalves belong to the second largest animal phyla Mollusca. Due to their aquatic life style and often limited mobility, bivalves have evolved a diverse repertoire of defence strategies to eliminate pathogens. The innate immune system of bivalves, including cellular and humoral responses, is one of the most important and sophisticated defence mechanisms among invertebrates. It utilises strategies such as phagocytosis, encapsulation, autophagy and humoral effectors for pathogen recognition and elimination (1, 2). One of these strategies also includes apoptosis, a type of programmed cell death, to prevent the spread of pathogens. Apoptosis leads to cell death of infected or unwanted cells, with cell shrinkage and nuclear fragmentation followed by phagocytosis of the apoptotic bodies by neighbouring cells, without needing to elicit an inflammatory response. Apoptosis is also involved in key developmental processes for organ differentiation and formation of structures in vertebrates and invertebrates alike (3). Apoptosis has been widely studied in molluscan species (4–6) and comparison with apoptotic pathways of pre-laterian, ecdysozoan (insects & nematodes) and vertebrate models have revealed that the complex

process of apoptosis in bivalve species shares many apoptosis-related genes with deuterostomes (Fig. 1) (6–8). By contrast, ecdysozoan apoptotic pathways such as in *Caenorhabditis elegans* and *Drosophila melanogaster* seem to be much simpler as a result of lineage specific gene losses.

Caspase-dependent pathways in programmed cell death

Although apoptosis requires a diverse group of proteins, receptors and enzymes, the key component of apoptotic pathways are caspases: protease enzymes that initiate and execute all other processes (9). In vertebrate models, two types of caspases exist: the initiator caspases (caspase-2, -8, -9, -10) and executioner caspases (caspase-3, -6, -7). Caspases are present in the cell as inactive zymogens containing a prodomain at the N-terminal and a large subunit (p20) followed by a small subunit (p10) towards the C-terminal. The prodomains of initiator caspases are often longer, containing homotypic interaction motifs such as the caspase-recruitment domain (CARD) in caspase-2 and caspase-9 and death-effector domains (DEDs) in caspase-8 and caspase-10 that function as recruitment domains. Caspases are cleaved by facilitating proteins to remove the prodomain and separate the large and small subunit at the intersubunit linker, which leads to the formation of a heterodimer of both subunits. To be activated, two heterodimers form a caspase dimer-complex (10, 11) with the catalytic histidine/cysteine dyad (active sites in p20 subunit) free to hydrolyse peptide bonds of target proteins (12, 13). Two major apoptotic pathways exist in deuterostomes and molluscan species: the extrinsic and intrinsic pathway (Fig. 1) (9, 14). The extrinsic pathway is activated by receiving apoptotic signals at the cell surface by transmembrane receptors, which then trigger the auto-catalytic activation of the initiator caspases-8. Activated caspases-8 cleave and activate the executioner caspases-3, -7 or -6, which regulate the final apoptotic events such as DNA fragmentation, plasma blebbing and proteolysis of key structural and cell cycle proteins including activation of additional executioner caspases (15). The intrinsic mitochondrial pathway is a non-receptor-mediated pathway with stimuli coming from various sources, for instance UV radiation, reactive oxygen species (ROS), mitochondrial DNA damage, viral infection and environmental pollutants (9, 14). In the centre of this proposed pathway are caspases-9, which form apoptosomes with apoptotic protease activating factor-1 (Apaf-1) and cytochrome c (Cyt c), and are regulated by various proteins associated with the mitochondria or in the cytoplasm. Inhibitors of apoptosis (IAPs) can also directly interact with the initiator caspase-9 (16), providing additional regulators to the intrinsic pathway. Caspase-2 is another initiator caspase, which potentially takes part in both apoptotic pathways as part of a PIDDosome or it can be activated via transmembrane tumour necrosis factor (TNF) receptor-related signals, but although this caspase is one of the most conserved members of the vertebrate caspase family and was one of the first identified, its actual pathways in the apoptotic process remains controversial (17).

Apart from apoptosis, caspases are also involved in an additional non-apoptotic cell death types, called pyroptosis, which is often linked to inflammatory response (18, 19). This pathway, mostly described in vertebrates, uses its own pro-inflammatory caspases (caspase-1, -4, -5, -11) usually including a CARD prodomain. After receiving signals from pathogen-associated molecular patterns (PAMPs) or a host-cell generated danger-associated molecular patterns (DAMPs), procaspases-1 form inflammasomes with

associated proteins via their CARD-domains. During this process caspase-1 is activated, whose proteolytic activity subsequently cleaves interleukins (e.g. IL-1 β or IL-18), cytokines important in cell signalling mediating the inflammatory response. Caspase-1 also cleaves Gasdermin D, an effector molecule that catalysis pyroptosis. Inflammatory signals can also be received by transmembrane toll-like receptors (TLR), which can initiate the transcription of interleukins for instance via a NF- κ B pathway or in relation to an caspase-8 complex that interacts with the inflammasome by cleaving interleukins or activating the NF- κ B pathway (20). In vertebrates, the response to lipopolysaccharides (LPS) is often mediated via caspase-4, -5 or -11, but it is unknown if this process also occurs in invertebrates via similar pathways.

Caspases in bivalves: an incomplete story

Besides being involved in the immune response, caspases also take part in developmental processes, including embryonal development in animals and humans as well as cell differentiation, proliferation, learning and dendric pruning among other functions (3). Several caspases have been identified in bivalve species with homologs to caspase-8 (21–25), caspase-2 (22, 26, 27), caspase-1 (27, 28), caspase-3 (26, 29–32), caspase-6 (32) and a potential bivalve specific group of caspase-3/7 (22, 33). Most of these bivalve caspases were assumed to be involved in apoptotic processes in relation to haemocytes responses to pathogen infections (21, 22, 25, 28, 29, 31, 34–36), environmental stressors (22, 24, 32, 33, 36) or developmental processes (26, 30). While caspase-related pyroptosis has not been investigated in bivalves, caspase-1 mediated pyroptosis has been suggested in other invertebrates such as sea cucumbers (37) and crustaceans (38). With caspases present in all bivalve species, it is realistic to assume that caspase-regulated apoptosis occurs in bivalves. However, crucial information on different caspase types in bivalves is still missing, for instance no caspase-9 homolog has been identified to-date, even though this caspase is central to all other apoptotic pathways. Furthermore, caspase-7 homologs have also not been reported, although predicted protein sequences in the National Center for Biotechnology Information (NCBI) database suggests such homologs for various bivalve species. Moreover, some discrepancies in caspase classification seem to have occurred. Identified Pacific oyster *Crassostrea gigas* caspase-1 (27, 28) displays an identical protein sequence to the identified *C. gigas* caspase-3 (29), while another caspase-3 homolog (30) differs from the prior mentioned caspase-3. Additional caspase-3/7 homologs in *C. gigas* (33) and the mussel *Mytilus galloprovincialis* (22) also suggests a bivalve-specific caspase group, thus indicating a much more complex caspase family present in bivalves than previously suggested.

To examine the caspase family in bivalves, we investigated the presence of caspase homologs using a combination of bioinformatics and phylogenetic analyses. We blasted the *C. gigas* genome for caspase homologs and identified 35 potential homologs in the addition to the already cloned caspases in *C. gigas* (21, 27–30, 33), *Crassostrea angulata* (26), *Crassostrea hongkongensis* (25, 31), *M. galloprovincialis* (22, 24), *Mytilus coruscus* (24), *Mytilus californianus* (23) and *Tegillarca granosa* (32). Phylogenetic analyses of these bivalve caspases, as compared to homologs in other invertebrates and vertebrate species, confirmed expansions of the initiator and executioner caspase groups while also suggesting a need to

correct some of the identifications of previously classified caspases. The identified homologs are discussed in relation to their potential implications for apoptosis, immune response and during development. The previously identified *C. gigas* caspases and an additional potential caspase-3 homolog were also used in an expression study in Pacific oyster larvae prior and after initiation of metamorphosis with the neurotransmitter epinephrine. Given that caspases are involved in such a wide variety of essential pathways, this analysis of caspases in bivalves brings new insight to their potential function, as well as correcting potentially misleading information from previous classification attempts. As such, we provide a solid foundation from which new directions can emerge that further our understanding of immune responses and developmental processes in bivalves.

Results

Phylogenetic assessment caspases in bivalves

Thirty-five new putative caspases have been identified in the Pacific oyster genome in addition to the twenty-three caspase homologs previously characterised in bivalve species (21–33). All identified bivalve caspases possess a large p20 caspase subunit unique for caspase homologues. However, ten of the newly identified *C. gigas* caspase homologues only contain a p20 subunit without a downstream small p10 subunit based on a conserved motif search with ScanProsite. Of all forty-six bivalve caspases with p20 and p10 subunits, nine bivalve caspase homologues contain CARDs in their prodomains, five have two DEDs in the prodomains, two homologues have an additional death domain (DD) motif after the two DEDs, four homologues have only one DED domain, as well as two homologs that have two caspase-unusual domains in their prodomain, the double stranded RNA-binding domains (DSRM). The remaining twenty-four caspases are relatively short without any specific domain in their prodomains. Trimmed CASc domains (caspase-specific domains, p20 and p10 subunits without intersubunit linker) of CARD or DED domains-possessing caspase homologues were aligned with known initiator caspases-2, -9, -8 and -10 of other species, as well as vertebrate inflammatory caspases-1, -4, and -5, which also contain CARDs. The remaining bivalve caspase homologues were aligned with known executioner caspase-3, -6 and -7 homologues.

The phylogenetic analysis of the CASc domains of initiator caspases included two clades, with one group including all CARD-containing caspases for caspase-2, caspase-9 and inflammatory caspases, and a second group that included DED-containing caspase-8 and caspase-10 homologues (Fig. 2A). In general, the initiator caspase divergence from the executioner caspases (outgroup Hs3 and Hs7) was highly supported in both phylogenetic analyses (Maximum Likelihood (ML) bootstrap percentage: 100%; Bayesian inferences (BI) posterior probabilities: 1.00). The CASc domains of CARD-containing initiator caspases revealed that the nine bivalve CARD-containing caspases showed the highest homology to caspase-2 homologues with no direct homologue found to vertebrate caspase-9 or the inflammatory caspase group. This classification was also supported by a separate phylogenetic analysis of the CARD domains (Fig. 2B), of which none directly grouped with either of the vertebrae caspase-9 or inflammatory caspase CARD clades. Furthermore, the p20 active site motifs of Ca2, Cg2, Cg2A (previously identified as

Cg2 (27)), Cg2B and Cg2C were identical to human caspase-2 homologue Hs2 with a QACRG motif and not to the human caspase-9 Hs9 motif QACGG (Fig. 2D). However, a QACRG motif is also present in inflammatory caspases, but based on the position of these bivalve caspases in the phylogenetic tree, it is less likely that these caspases were homologs to the inflammatory caspase, although similar functional characteristics cannot be excluded. The remaining bivalve caspase-2-like homologues displayed very different p20 motifs, although the three Cg2-like homologues contained the conserved cysteine in this motif. However, in contrast to the other initiator caspases, Cg2-like C contained an arginine instead of the conserved histidine residue ahead of the p20 motif QARXG. An outlier to all proposed bivalve caspase-2 homologues was the *M. galloprovincialis* caspase-2 homolog Mg2-like (previously identified as Mg2 (22)), which neither containing the conserved cysteine or histidine residue.

The second clade containing caspase-8 and caspase-10 homologs possessed eleven of the identified bivalve caspases of which three (Cg8-like A-C) were newly identified in the *C. gigas* genome (Fig. 2A). Rather than being direct homologs to either vertebrate caspase-8 or caspase-10 members, they grouped outside the vertebrate caspase-8/10 group in three small groups: caspase-8A, caspase-8B and a caspase-8-like group. The bivalve caspase-8A group was clustered together according to their species genus based on the three *Mytilus* caspase-8As and the two *Crassostrea* caspase-8As. The two bivalve caspase-8B homologues, Cg8B (previously described as Cg8 (21)) and Mc8B, containing two DEDs and a DD domain in their prodomains, also grouped together based on their CASc domain sequence. Similar phylogenetic arrangements were seen for the DED domain analysis (Fig. 2C). The four bivalve caspase-like homologues, Cg8-like A-C and Mg8-like (previously identified as Mg8 (22)), however, were less conserved in direct comparison to the CASc and prodomain phylogenetic positions, and only contained one DED each. Nevertheless, with one DED in their prodomains, and the conserved caspase-8 p20 motif QARQG (except for Cg8-like C motif QICQG) present, supported by their position in the phylogenetic tree, these four bivalve caspases are likely homologues of caspases-8/10.

The phylogenetic analysis of the executioner caspases CASc domains showed a more complex relationship within this type of caspase, as well as more variety in the p20 active sites QXCXG (Fig. 3). Although highly supported by BI analysis (posterior probabilities: 0.78) as a clustering group to the outgroup Hs8 and generally highly supported in terms of direct homologs within the executioner caspase clade, positionings of the larger subclades were generally poorly supported by both analyses (Fig. 3A) and resulted in polytomy in the BI analysis. Thus, positioning of these subclades might change, when new information on additional executioner caspases will emerge in the future. Nevertheless, the phylogenetic analysis of the potential bivalve executioner caspases revealed distinct clustering of the thirty-six bivalve caspases, with some clades potentially unique to bivalves. None of the bivalve caspases showed direct homology to either of the vertebrate groups (caspase-3, caspase-7 or caspase-6) or the clade of arthropod caspases. The two bivalve caspases, Cg3A and Tg3A (previously described as Tg3 (32)) grouped outside the vertebrate caspase-3 and caspase-7 clade. Interestingly, although Cg3A and Tg3A seem to be the closest homologs to vertebrate caspases-3/-7, both p20 active sites varied in their amino acid sequence (Cg3A: QSCRG, Tg3A: QTCRA) compared to the commonly found vertebrate QACRG sequence (Fig. 3B). Moreover, the histidine residue in Cg3A was not conserved and contained a tyrosine

at this position instead. Alignments of the full coding sequence (CDS) and protein sequences of the previously cloned *C. gigas* caspase homologs caspase-3 (29), caspase-1 (27), and caspase-1 (28) showed that these caspase homologues are indeed nearly identical (Additional file 1) and appear to be different isoforms of the same caspase. For our phylogenetic analysis, the isoform identified in the *C. gigas* genomes was considered to be representative of this caspase (CASC domains 100% identical) and hereafter named Cg3B based on its position in the tree (Fig. 3A) and its active p20 sites and conserved residue (..H...QACRG), which is identical to the active sites in human executioner caspases. Cg3B did not show high homology to the inflammatory caspase group caspase-1 or the executioner arthropod caspase clade including arthropod caspases-1. A second Cg3B-like caspase was identified with moderate CASC sequence identity to Cg3B (74% identity). However, a deletion event appeared to have occurred in Cg3B-like leading to the loss of the arginine and glycine residues in the p20 active site QACRG. Cg3C and Ca3C (previously identified as Cg3 (30) and Ca3 (26)) were grouping together with two additional novel *C. gigas* caspases Cg3C-like A and Cg3C-like C, which contained the unique two DSRM domains in their prodomains. These bivalve caspase-3 like homologues were homologues to the previously identified deuterostome caspases Aj3 (echinoderm *Apostichopus japonicus* (39)) and B13 (amphioxus *Branchiostoma lanceolatum* (40)). Three subclades of caspase-3-like bivalve caspases were also identified. The largest group contained caspase-3/7 homologs, clustering further outside the vertebrate caspase-3 and caspase-7 groups as well as the bivalve caspase-3A group and the cnidarian caspase-3 homologs, Av3 (41) and Ep3 (42). This group contained members of several bivalve species with Ch3/7, Tg3/7, Mg3/7 and Cg3/7 (all previously identified as Ch3 (31), Tg6 (32), Mg3/7_3 (22) and Cg3/7 (33)) as well as eleven newly identified *C. gigas* caspases Cg3/7A-L. Conserved residues and active sites in the p20 subunits are mostly preserved with ..H.....QACRX only varying in the last residue of the active site. Cg3/7K and Cg3/7L did not contain a p10 subunit. A second large group named caspase-3/7-like, as it positions even further from vertebrate caspase-3 and caspase-7, only comprised newly identified *C. gigas* caspases, with most of them containing only a p20 subunit and highly diverse p20 active sites. Cg3/7-like was the only member of this group that contained the conserved histidine in the p20 subunit. The third group combined three previously identified Mg3/7 homologs, for which previous phylogenetic analysis already showed unique clustering within executioner caspases together with a caspase-3 homolog in *Hydra vulgaris* Hv3A (22, 43). However, other bivalve homologues to this group were not identified in the current analysis, and naming of these mussel caspase homologs remain as previously reported.

Gene expression: caspases during larval development and metamorphosis

The gene expression profile of the five previously cloned Pacific oyster *C. gigas* caspases, Cg2A (27), Cg8B (21), Cg3/7 (33), Cg3B (27–29) and Cg3C (30), as well as the best hit for a caspase-3 homolog in our BLAST search of the *C. gigas* genome, Cg3A, were assessed during late larval development, after induction of metamorphosis and in spat to investigate if these caspases are potentially involved in the metamorphic transition from larvae to spat in the Pacific oyster. Induction of metamorphosis in

competent oyster larvae was successfully achieved after exposure to the well-known metamorphosis inducer epinephrine (44) for 3 h with epinephrine resulting in $81.3 \pm 2.2\%$ metamorphosis compared to $3.1 \pm 0.3\%$ metamorphosis in non-treated animals ($p < 0.01$). All caspase genes were expressed in all larvae and spat sample points displaying unique expression profiles. The expression of initiator caspase Cg2A significantly decreased in larvae 14 days post fertilisation (dpf) to 17 dpf, a time during which larvae reached competence for metamorphosis, and again after 3 hours post exposure start (hpe) and 6 hpe to epinephrine. In spat (24 hpe), Cg2A expression significantly increased again to a level comparable to that observed prior to induction of metamorphosis. The second proposed initiator caspase, Cg8B, on the other hand, increased its expression just prior to competence in 16 dpf, but expression then decreased during and post metamorphosis. The four executioner caspases also displayed slight differences in their expression profiles, with Cg3/7 expression significantly decreased throughout larval development and after metamorphosis induction, and remained low in spat. Cg3A expression was steady during development, but decreased after 6 hpe with a significant decrease observed in spat. Cg3B showed a similar profile as Cg3A, but expression increased slightly during metamorphosis, followed by a significant decrease in expression for spat. A decrease in expression throughout development and metamorphosis was also seen for Cg3C, but contrary to the other executioner caspases, Cg3C expression increased again in spat.

Discussion

Caspases in bivalves

Caspases are key players in the apoptotic and inflammation processes in most vertebrates and invertebrates, hence the diversity of caspases present in bivalve species is not surprising. The phylogenetic relationship of the fifty-six previously and newly identified bivalve caspases is relatively complex, and shows that bivalves possess caspase homologues that are different from the well-known vertebrate caspase groups, with expansions in both the initiator and executioner caspase groups. Interestingly, bivalves, and in particular *C. gigas*, contain several caspases where the histidine and cysteine residues in the p20 subunit were not conserved. These bivalve caspases may have lost their catalytic function, given that histidine and cysteine are essential for this process (13). Furthermore, some of the newly identified *C. gigas* caspases do not contain a p10 subunit, which has been previously described for metacaspase-like proteins of non-metazoans with metacaspases being caspase homologues present in prokaryotes up to the level of higher plants (45, 46). While the p10-minus non-metazoan caspases are suggested to be non-functional, or potentially vary in their substrate specificity to traditional caspases (46), further research is needed to clarify whether these unusual bivalve caspases are functional specifically in combination with histidine/cysteine residue mutations.

Bivalves possess a variety of initiator caspases, with either CARD or DED motifs present in their prodomains. The absence of a direct caspase-9 homologue is particularly notable. None of the identified bivalve caspases possessed the caspase-9 typical p20 active site QARGG. A caspase-9 homologue has not been characterised in any bivalve species to date. Only expressed sequence tags (ESTs) in *C. gigas*

(27) and Manila clams, *Ruditapes philippinarum* (47) have been identified as caspase-9 homologues by automatic annotation without detailed analysis of sequences or phylogenetic relationships, and ESTs annotated as caspase-9 homologues were not described for the oyster *Ostrea edulis* (48). It appears that bivalves lack this type of caspase, which seems surprising given that caspase-9 plays an important role in the intrinsic apoptotic pathway forming apoptosomes with Cyt-c and Apaf-1 proteins for further downstream activation of executioner caspases (Fig. 1). Additional bivalve genomes need to be searched for potential caspase-9 homologs to shed light on this missing homologue. Interestingly, caspase-9 activity was detected in *C. gigas* haemocytes after UV irradiation that initiated apoptosis in haemocytes by using a vertebrate caspase-9 activity assay (49). Caspase-9 activity was increased in the presence of *C. gigas* Cyt-c and inhibited by vertebrate caspase-9 inhibitor Z-LEHD-FMK, suggesting that a caspase-9 like homologue is indeed expressed in the Pacific oyster. This study also proposed that caspase-9 activity is required for successful caspase-3 activation, thus supporting the idea that the caspase homologue's activity, in response to the vertebrate caspase-9 activity assay and inhibitor, operated upstream of any caspase-3-like activity during apoptosis in oyster haemocytes. It is possible, that one of the identified *C. gigas* caspase-2 homologues might function in a caspase-9 like manner, given that *C. gigas* possesses seven caspase-2 or caspase-2 like homologues, which is more than is reported for any other species. Further research is needed to support this theory, and verify if one of these *C. gigas* caspase-2 homologues operates similarly to vertebrate caspase-9, including the ability to hydrolyse LEHD sequences of substrates, which seems to be specific for vertebrate caspase-9 homologues (12). Apoptosome formation with Cyt-c, an initiator caspase and Apaf-1 protein already appears to differ in bivalves from the vertebrate apoptosomes, wherein research has shown that Apaf-1 proteins in bivalves are missing CARD domains and only contain WD-domains for binding Cyt-c (49).

With several caspase-2 homologues present in bivalves, this expansion of caspases might take part as initiators of programmed cell death along the two apoptotic pathways. As mentioned previously, the vertebrate caspase-2 pathways are not fully resolved, but caspase-2 s form PIDDosomes by binding to cytoplasmic p53-induced proteins with death domains (PIDD) and bipartite adapter RIP-associated Ich-1/Ced-3-homologue proteins with death domains (RAIDD) as part of the intrinsic apoptotic pathway (17). PIDD protein homologues have been reported in the genome of the clam species *Mya arenaria* (7) and *R. philippinarum* (47). A RAIDD homolog has also been identified in the mussel *Mytilus coruscus* in the NCBI database (GenBank ID: CAC5373468.1), thus supporting functional bivalve caspase-2 homologues including formation of PIDDosomes. RAIDD adaptor binding to caspase-2 might also be involved in a more direct response with TNF-receptors for the extrinsic pathways interacting with executioner caspases. Caspase-2 regulated apoptosis in vertebrates has implications for host-pathogen interactions as well as in responses to endoplasmic reticulum stress and DNA damage (50). Bivalve caspase-2 homologues show similar implications with upregulated expression of Mg2-like and Cg2B in haemocytes after UV treatment (22) or in bacterial challenges (27), respectively. Increase in gene expression of Mg2-like also suggests that even caspase members without the conserved sites in the p20 subunit might fulfil an essential function during immune response, although it is doubtful that this caspase homologue has maintained a catalytic function. The surge in *C. gigas* caspase-2 homologues also indicates that more

caspase-2 homologues might exist for *Crassostrea angulata* and *M. galloprovincialis* than the two previously characterised, in particular as these two are too distantly related to be the only representatives of this caspase-type in their species.

One of the most conserved caspase groups is the initiator caspase-8 group containing DED motifs in the prodomain. This group is also highly conserved in bivalves and several caspase-8 homologs have been previously characterised. Our BLAST search only identified four new *C. gigas* caspase-8 homologues, which were grouped together with the already characterised bivalve caspases-8. To transmit apoptotic signals in vertebrates, caspase-8 forms a complex with membrane-bound death receptors (i.e. Fas, TNF or TRAIL) binding to Fas-associated proteins with death domain (FADD) via a DD motif. This complex recruits procaspases-8 via a DED motif between FADD and the caspase prodomain. Caspase-8 then undergoes self-cleaving for activation. Many of these death receptor homologues have been described in bivalve species (1, 27, 51–60) as well as FADD homologs in different oyster species (27, 48, 53). Based on previous research, caspase-8 homologues seem to be functional and operate in a vertebrate-like manner. Ch8A (25) and Cg8B (21) were both located in the cytoplasm after cell death was induced. Furthermore, Ch8A (25) and Mc8A (23) were able to activate human caspase-3 in transfected cells, supporting a downstream activation of executioner caspases by oyster and mussel caspase-8 homologues. Overall, we identified three types of caspase-8 homologues in bivalves: (1) caspases with two DED motifs; (2) with two DED motifs and an additional DD motif, and (3) with only one DED motif in their prodomains. How these variations in prodomain motifs affect their binding ability to FADD or death receptors is not clear. In vertebrates, FADDs can also interact with only one of the caspase-8 DEDs (61), suggesting that one-DED-containing caspase-8-like homologs of bivalves are potentially able to bind to FADD. An analysis of the binding ability of Mc8B, the mussel homolog containing two DEDs and one DD motif, showed that the Mc8B DD motif is able to bind to the DD motifs of the human Fas death receptor and human FADD adapter protein (23). Thus, this additional DD motif might provide an opportunity to directly bind to Fas and/or FADDs. We also identified a DD motif in Cg8B caspase that was not previously reported (21), suggesting that the additional DD motif is potentially common in this bivalve caspase-8 B group. In mammals, the second DED motif in the caspase-8 prodomain has a role in inhibiting apoptosis by inactivating procaspases-8/FADD complex through binding to a DED motif containing inhibitor c-FLIP (62). Although no FLIP homologues have been identified in bivalves, the second DED motifs in caspase-8A and 8B groups might still be utilised for apoptosis regulation. Interestingly, FLIPs (v-FLIPs) have been identified in herpes viruses as a viral inhibitor with v-FLIPs inhibiting apoptosis of host cells when infecting cells (63). Thus, the presence of only one DED motif containing bivalve caspase-8 homologues might be an adaptation to viral infection that utilises v-FLIPs to prevent host cells of apoptotic immune responses.

In *C. hongkongensis*, Ch8A has been shown to activate the NF- κ B transcription factor (25). The NF- κ B pathway in vertebrates is involved in the transcription of pro-inflammatory cytokines (pro-interleukins IL) and cytoplasmic Nod-like receptors (NLRPs), which take part in the pyroptotic inflammatory response together with caspase-1 and other inflammatory caspases (19). The FADD-caspase-8 complex is also involved in activating and regulating inflammation (20), thus, it is likely that bivalve caspase-8

homologues are also potentially involved in inflammation responses. However, cleaving of interleukins and induction of pyroptosis by inflammasomes including caspase-1 might differ between bivalves and vertebrates given that our BLAST search of the *C. gigas* genome did not identify an inflammatory caspase homologue (caspase-1/-4 or -5) nor was one reported previously for any other bivalve species. The previously characterised *C. gigas* caspase-1 homologues (28) did not show any characteristics of vertebrate caspase-1. This *C. gigas* caspase homologue was also not able to cleave the tetrapeptide substrate sequence YVAD, which is specific to vertebrate caspase-1. Instead it was able to cleave DEVD and DMQD tetrapeptide sequences, which are specific to caspase-3-like caspases. This *C. gigas* caspase was renamed Cg3B in our study. Thus, inflammatory responses via pyroptosis might be regulated differently in bivalves, although we cannot exclude that a different caspase homologue has taken over this function. In general, pyroptosis in invertebrates related to caspases has only been reported in a few cases. For instance, caspase-1 homologues, Mj1 and Aj1 with pyroptotic-like functions were reported in *Marsupenaeus japonicas* shrimp in relation to the white spot syndrome virus infection (38) and in the *A. japonicus* sea cucumber in relation to *Vibrio splendidus* challenge (37). Although both caspases do not contain a CARD motif in their prodomain, as is common for vertebrate inflammatory caspases, both caspase-1 homologues showed caspase-1 like activity and response to YVAD substrates as well as binding to IL-1 β like proteins in shrimp. Nevertheless, our phylogenetic analysis clustered both caspase homologues with the executioner caspase clade, and none of our identified bivalve caspases showed close phylogenetic relationships with these two caspase-1 homologues, suggesting that bivalve, crustacean, echinoderm, as well as vertebrate inflammatory responses, have evolved differently.

The expansion of the executioner caspases in bivalves provides numerous caspase homologues for potential apoptotic regulation and execution, including possible involvement of several executioner caspases with distinct functions. In vertebrates, executioner caspases such as caspase-3, caspase-6 and caspase-7 are also functionally distinct, interacting with each other, cleaving different substrates, or displaying different cleaving efficiencies for the same substrate (64). However, bivalve execution of apoptosis seems to differ from the vertebrate model given that no direct homologs to vertebrate's executioner caspases have been identified. Instead, bivalve appear to possess their own specific groups, which may in some cases even be bivalve-specific. The bivalve caspase3A group, which is the most closely related group to vertebrate caspase-3 and -7, varies in the p20 conserved active site. However significant changes in gene expression patterns of Cg3A during development (Fig. 4), and Tg3A after cadmium exposure (32) were observed, thus suggesting that these two caspases might still fulfil essential functions in both development and immunotoxicity responses. Most prior research was conducted on Cg3B, for which no caspase homologue in another bivalve species has been identified. This *C. gigas* caspase was cloned several times independently, with different names assigned (27–29); it was renamed Cg3B in this study. Additional studies on Cg3B have been conducted in relation to spatial distribution (caspase-1 (30)) and in response to bacterial challenge and development (caspase-3 (34)). A study in *C. gigas* haemocytes on FMRFamides, specific neuropeptides, assessed the expression of Cg3B after stimulation with CgFMRFamides twice as caspase-1 and caspase-3 based on the primer pair sequences provided (35), with both analyses showing significant increases of Cg3B an hour after

FMRamide injection. Cg3B is expressed in the cytoplasm (28, 30) as well as in the nucleus (28), which is similar to vertebrate caspase-3 translocating from the pro-form cytoplasm to an active form in the nucleus (65). Cg3C, on the other hand, was only detected in the nucleus. Cg3B and Cg3C also displays high proteolytic activity to DXXD like substrates, which is similar to vertebrate caspase-3 homologues (28–30), as well as Cg3B induced apoptosis insomuch as it cleaves the poly ADP-ribose polymerase (PARP), a DNA repair protein (29). In vertebrates, caspase-3 cleaves PARPs to inhibit DNA repair and induces apoptosis (64). Furthermore; Cg3B display LPS binding activity, although some discrepancy in the LPS recognition was described with one prior study supporting binding LPS with the N-terminus (29), while another previous study supported the C-terminus (28) of this caspase homologue.

Localisation of Ch3/7 in cytoplasm and nucleus in HEK293T cells as well as apoptosis activity of Ch3/7 in haemocytes was confirmed with Ch3/7 RNAi decreased apoptotic rates and Ch3/7 expression (31). Ch3/7 are also able to activate the NF- κ B pathway and p53 pathway. p53 is of particular interest when cells experience stressful environmental conditions such as DNA damage, UV light or tumorous growth with p53 activation inducing apoptosis by blocking specific cell cycle pathways (66). p53 members were identified in several bivalve species in relation to apoptosis and neoplasia (7, 67–69). p53 pathway activation was also found by Ch8 homologue with an intermediate activation of human caspase-3 (25), suggesting that both *C. hongkongensis* caspases regulate apoptosis via a p53 pathway. Caspase-3 like activity was also reported for Cg3/7 with DEVDase activity (33), for which research suggested that the long intersubunit linker sequence of Cg3/7 found in many caspase-3/7 members is essential for maximal DEVDase activity. Increases in expression of Mg3/7 (22) and Tg3/7 (32) after apoptosis induction with UV light or cadmium further supports that this large bivalve caspase group is important during apoptotic processes. Nevertheless, how the newly identified Cg3/7 members and the Cg3/7-like group are involved in apoptosis still needs to be clarified. Cg3/7A-J caspases contain the conserved histidine/cysteine residues for a potential functionality, but Cg3/7-like homologues are less conserved, and sequences seem to be unique, which might suggest a potential new type of caspase in metazoans. Furthermore, two Cg3C-like homologues with DSRM motifs in their prodomain were identified, which is a motif that has not been previously reported for any caspase. DSRM motifs are usually used for posttranslational modifications of proteins, but they could also potentially take part as sensors and modulators of innate immunity (70) by recognising intracellular viral dsRNA. The DICER protein is one of the better known examples of a DSRM motif-containing protein that recognises virus-derived RNA and is capable of degrading the dsRNA to small interfering siRNA or mircoRNA (71). A CgDICER homologue has been identified, including DSRM motifs, which was able to bind to viral dsRNA and poly(I:C) (a synthetic viral dsRNA analogue) (72), supporting the idea of functional DSRMs for viral pathogen detection in bivalves.

Caspases in bivalve development, immune system function, and stress-responses

The role of caspases and apoptosis in organogenesis and embryonic/larval development is well studied for many invertebrate species (73, 74), and expressions of various initiator and executioner caspases have also been reported throughout embryonic and larval stages in bivalves (21, 26, 27, 30, 34). Caspase-

regulated programmed cell death might also play a role in metamorphosis, which marks the transition from a larval to juvenile life stage. During metamorphosis, bivalve larvae undergo a massive restructuring wherein they lose larval organs such as the velum and foot (species specific) that are required for pelagic dispersal, in favour of adult organs such as gills that are suited to later more sessile life stages. The loss of larval organs is almost certainly regulated by caspase-dependent apoptosis, as seen for many other animals that undergo metamorphosis (73, 75, 76). Our analysis of *C. gigas* caspases prior, during and post metamorphosis (spat, also called juveniles 24 h post metamorphosis induction) showed expression of the proposed initiator and executioner caspases. However, although each caspase displayed a unique expression pattern, none of the six tested caspases showed an upregulated transcription during the first 6 hpe after the metamorphosis-inducing epinephrine treatment, with most of the larvae displaying a decrease in their expression (Fig. 4). Several possible explanations may elucidate these findings. First, apoptosis during development could be a very localised event in certain tissues, as compared to apoptosis during an immune response which is very rapid, intense and unspecific. Apoptosis during metamorphosis might be slower and thus might not be significantly reflected in transcription patterns. Furthermore, caspases are usually present in the cell as inactive zymogens until needed; thus, a slow accumulation could take place over time. Moreover, our sampling time point might have been too early to capture the full apoptotic process for larval organ absorption. A previous study on caspases during metamorphosis in *C. gigas* showed that Cg3B and Cg3C are highly expressed 6–24 h and 6–48 h post settlement, respectively (30). Although it is not specified how these authors defined ‘post settlement’ (metamorphosis is a gradual process that occurs over a period of 12–36 hours), these expression patterns suggest caspase activity occurs late in the metamorphosis process. Caspase homologs in *C. angulata*, Ca2A and Ca3C, also showed expression peaks 6 hours post metamorphosis, although once again the authors did not define how they determined ‘post metamorphosis’ which is problematic as a specific timepoint in the transition process would be difficult to identify, especially in a 6 h window. *In-situ* hybridisation of these two oyster caspases in larvae prior to metamorphosis suggested the presence of caspases in the velum and larval foot (77). Finally, it is also possible that none of our six caspases are involved in metamorphosis, as indicated by their decline in expression. Further investigations regarding caspase expression and regulation during bivalve metamorphosis are needed to gain more insight into how caspases are involved in the transition from larval to spat. However, as our gene expression profile of the four different executioner caspases, Cg3A, Cg3B, Cg3C and Cg3/7 clearly demonstrated, selection of the specific caspase-3 homologue to investigate is important, as these can vary through significant down- or upregulation before and after metamorphosis.

The implications of programmed cell death and additional inflammation responses have also been widely discussed in relation to the bivalve innate immune system (1, 2, 4, 6, 78–80). Haemocytes are one of the crucial executioners of invertebrate immune responses, and one of the proposed key strategies of haemocytes is to undergo apoptosis when infected by a pathogen, or when they have ingested invaders through phagocytosis to prevent proliferation and spread. Pathogens on the other hand, are seeking tactics to prevent apoptosis, for instance by inhibiting enzymes such as caspases, or through strategies that avoid triggering the host cell response. Thus, caspase expression, as well as their presence and

activity in haemocytes or tissues where pathogens are present, is of great importance to our understanding of host immune defence, as well as our understanding of pathogens. Spatial expression studies of caspases in mussels and oysters have shown that initiator and executioner caspases are frequently expressed in haemocytes as well as gills, digestive glands and labial palps, which are areas exposed to pathogens and environmental stressors (i.e. pollutants) (21, 22, 28, 30, 31, 81). Moreover, our identification of a large number of different bivalve caspases with unique traits and motifs such as lack of second DED, or additional DDs and DSRMs in prodomains, or variations in p20 active sites, provides insight into the regulatory mechanism of apoptosis. It also provides information about potential adaptations involved in pathogenesis. Furthermore, exposure of different bivalve species to pathogens such as viruses, bacteria and parasites has shown recruitment of apoptosis-associated proteins and enzymes (22, 34, 48, 82, 83). These include different effects on transcription of caspases to LPS, poly I:Cs, lipoteichoic acid of gram-positive bacteria, CpG of *Vibrio* sp., zymosan (yeast glucan) as well as common pollutants (PAHs and PCBs) (22). Exposure of *C. gigas* embryos and larvae to the gram-negative bacteria *Vibrio coralliilyticus* has shown that apoptotic responses to pathogens develop early in oyster larvae (blastula – D-shelled larvae) near the digestive gland with first Cg3B appearing in D-larvae 12 h post exposure to bacteria (34). *V. splendidus* also increased caspase-3 activity in the posterior adductor muscle in *M. galloprovincialis* 24–72 h post injection (84). Furthermore, *Vibrio alginolyticus* increases the expression of Ch8A and Ch3/7 in haemocytes of *C. hongkongensis* oysters (25, 31). Interestingly, the same *Vibrio* sp. did not induce the expression of the *C. gigas* caspase-8 homologues in Pacific oyster haemocytes after bacterial challenge (21). This could either be a species specific or a caspase-type specific response. Based on our phylogenetic analysis, Ch8A and the assessed oyster caspase-8 homolog, here classified as Cg8B, belong to two different types of bivalve caspase-8 homologs with potentially different functions and responses to pathogens. LPS from *Escherichia coli*, another gram-negative bacterium, has been shown to inhibit activity of Cg3B in *C. gigas*, thus causing a reduction of apoptosis in HEK293T cells (29). The authors concluded that Cg3B potentially functions as a target, or even as sensor of LPS, with inhibition of apoptosis evoked by the host as a survival strategy. LPS and *Vibrio* spp. exposures have also resulted in an increase in IAP homologs in *C. gigas* (27, 81) and *Ruditapes decussatus* (85), with IAPs known inhibitors of apoptosis. CgIAP2 has also been shown to inactivate Cg2A (81). However, the same study also demonstrated that *C. gigas* possesses 48 potential IAPs, more than for any other species reported. However, it is still not clear exactly how IAPs affect apoptosis in bivalves, in particular since no caspase-9 homologue for apoptosome formation has been identified yet. The mitochondrial protein Smac/Diablo is known to inhibit IAPs (86); and a CgSmac homolog in *C. gigas* haemocytes was significantly upregulated after LPS exposure (72). When CgSmac was suppressed, apoptotic rate in haemocytes, as well as caspase-3 activity, decreased. In contrast, LPS also induced the expression of regucalcin (RGN) in *C. gigas*, a regulator of calcium homeostasis in haemocytes, and when CgRGN was suppressed, the apoptosis rate in haemocytes increased after LPS stimulation (87). Thus, LPS and *Vibrio* spp. can affect apoptotic genes such as caspases, IAPs, mitochondrial proteins and other participants in the haemocytes of hosts, with complex effects on apoptosis execution. IAPs also seem to be upregulated in response to Ostreid herpesvirus type 1 (OsHV-1) in *C. gigas* (88), while Cg3B expression is downregulated in infected haemocytes. It is assumed that this

is a virus counter measure to the Pacific oyster's response to viral infection (89, 90) given that OsHV-1 is known to suppress apoptosis in *C. gigas* haemocytes (90, 91). Inhibition of apoptosis also plays a role in parasitic infections, as proposed for *Bonamia* spp. which are highly infectious in the European flat oyster *O. edulis*. Comparison between wild populations and oysters selected for resistance showed that inhibition of apoptosis, and an increase in IAP expression, predominates in the wild oysters (82). Thus, apoptosis of infected cells or haemocytes, which have phagocytosed parasites, can prevent the spread of this pathogen (82, 92). Gervais and colleagues (48, 93) reported that apoptosis in haemocytes occurred in *O. edulis* 1– 4 h post *Bonamia* contact, and continued to induce the expression of apoptotic genes many days after infection, but with differences between individuals originating from different locations in France. In relation to caspase involvement in *Bonamia* infection of *O. edulis*, it is noteworthy that based on our phylogenetic analysis and *O. edulis* transcriptome data (94), the proposed flat oyster caspase-3 homologue (93) seems to be an homologue to Cg3A (94% identity) with identical p20 conserved sites ..Y....QSCRC. However, the proposed caspase-2 homologue did not resemble any of our bivalve caspase-2 homologues, but showed 77% identity with CgCARDDCP-1 genes instead. This *C. gigas* CARD-containing protein is not a caspase, but nevertheless seems to be involved in LPS binding and activation of NF- κ B pathway after *Vibrio* stimulation (95).

Caspase activation and regulation in relation to apoptosis can also be impacted by environmental stressors, for instance temperature, oxygen and heavy metals. In *Mytilus* spp., extreme temperatures can lead to DNA damage, reduction in haemocyte variability and lysosomal membrane stability as well as activation of apoptotic proteins (24, 96, 97). *M. galloprovincialis* Mg8A and *M. coruscus* Mco8A expression and activity increased significantly in gill tissues and haemocytes after exposure to extreme heat and cold (24). Furthermore, caspase-3 activity was upregulated in haemocytes of *M. galloprovincialis* and *M. californianus* after high and low temperature stress (96). Hypoxia (oxygen deficiency) and reoxygenation (H-R) in *M. edulis* and *C. gigas* also lead to increases in upregulation of genes related to apoptosis, autophagy and inflammation (36). *C. gigas* exhibited a higher hypoxia tolerance, which was also expressed in lower and/or later expression of apoptotic and inflammatory genes. For instance, when considering the chosen caspase-3 homologues based on our phylogenetic analysis members of the caspase-3/7 group, Mg3/7 expression increased after 1– 6 h H-R cycles, while Cg3/7 expression was not affected. However, it cannot be excluded that Cg3/7 is not the executioner caspase involved in hypoxia-related apoptotic responses, and that another executioner caspase might be responsible given that *C. gigas* possess many alternatives (as we point out, 39 in total). Nevertheless, this study also showed that Mg2-like and Mg8-like caspases, two of the lesser conserved bivalve caspases, were upregulated after an 1 h H-R cycle, suggesting that these unconventional initiator caspases are potentially functional and involved in H-R responses. Heavy metals such as cadmium also affect apoptosis performance in haemocytes of oysters (98) and clams (32). Caspase-3 activity was not recorded after cadmium-exposure in the haemocytes of the oyster *Crassostrea virginica*, thus potentially suggesting that apoptosis was induced by an caspase-independent pathway (98); transcription of Tg3A and Tg3/7 in the blood clam *T. granulosa* was also impaired, indicating a dysfunction of apoptosis in haemocytes (32).

Conclusion

Apoptosis in bivalves is a complex process, where most of the participating proteins, interactions and pathways remain unidentified. Our phylogenetic analysis of the twenty-two previously identified bivalve caspases, as well as the thirty-five previously unidentified *C. gigas* caspases based on our blasting of the Pacific oyster genome, shed light on the complexity of apoptotic and inflammatory pathways in this class of molluscs. In contrast to previous theories, bivalve apoptotic and inflammatory pathways appear to differ even further from the well-characterised vertebrate pathways, for instance with an absence of essential caspase homologues such as caspase-9, caspase-1 or direct homologues to caspase-3, -6 and -7. Moreover, bivalves possess additional members of existing and novel caspase groups for both initiator and executioner caspases, including unique motifs in the caspase prodomains, further supporting a more unique function of caspases in regards to programmed cell death. This could be of particular interest in relation to the function of the bivalve immune system. Without the complex adaptive immune system that is present in vertebrates, most protostomian invertebrates have to rely on other regulatory mechanisms to regulate their innate immune responses to pathogens. The presence of several unique caspases could provide an additional regulatory instrument to respond to the large variety of pathogens, but also to environmental stressors that bivalve, especially more sessile species are increasingly exposed too. The presented phylogenetic analysis of bivalve caspases also provides a foundation for additional caspase-related research in bivalves or other lesser studied invertebrates. Automated annotation and poorly conducted phylogenetic analysis, including insufficient numbers of characterised homologues, can lead to inaccurate classification of newly identified proteins and incorrect assumptions about potential functions. As such, we have shown that caspases in bivalves are substantially more multifaceted than was previously understood.

Material And Methods

Identification bivalve caspases and phylogenetic analysis.

Putative *C. gigas* caspases were identified by using tBLASTn and BLASTp searches of the Pacific oyster genome at NCBI (genome annotation release102) with protein sequences of human caspases (caspase-1 to -10) used as template. Identified protein sequences were checked for caspases-specific domains by Conserved Domain Database at NCBI (99) and ScanProsite (100). Each identified protein sequence was used in a BLAST search against vertebrate protein database to exclude homology to possible non-caspase proteins.

The large p20 and small p10 domains of the 35 putative *C. gigas* caspases identified by a BLAST search, together with the 23 previously identified caspases in bivalves, were aligned with caspases homologs (caspase-1 -caspase-10) of vertebrates and other invertebrate species using the default parameter of MUSCLE v3.8.31 (101) and edited manually in case of errors. Representatives of caspase homologs to vertebrate caspase-11 – caspase-16 or tunicates caspase-17 – caspase-22 were excluded as previous research has shown that these caspases are specific to these subphyla (102, 103). For those putative

caspases, which did not contain a small p10 domain, remaining protein sequence downstream to the large p20 domain were used in the alignment instead. A full list of all protein sequences used in the BLAST search and phylogenetic analysis are provided in Additional file 1. The alignment was trimmed with the intersubunit linker and insertions of single proteins were removed (Additional file 2 & 3). Two main trees were constructed for the proposed initiator and executioner caspases, pre-selected based on presence of CARD and/or DED containing prodomains, and a rough preliminary maximum likelihood tree containing all sequences. Two separate trees were constructed using the full sequence of CARD or DED domains (alignment Additional Files 4 & 5). For each tree a Maximum Likelihood (ML) and a Bayesian Inference (BI) analysis were constructed. Models of protein sequence evolution for ML (AIC criteria) and BI (BIC criteria) analyses were estimated with ProtTest v2.4 (104) including proportion of invariable sites (+ I), amino acid frequencies (+ F) and gamma shape (+ G): matrices LG + I + G + F (ML & BI initiator caspases, ML DED domains), LG + G (ML & BI executioner caspases, BI DED domains) and Blosum62 + G (ML & BI CARD domains). The ML analyses were constructed using PhyML v3.1 (105) and 1,000 bootstrap replicates. The BI trees were calculated using MrBayes v3.2.7 (106) with four randomly started simultaneous Markov chains running for two million generations, chains sampled every 100 generations and a burn-in of 5000 trees.

Animal husbandry and metamorphosis assay

Pacific oyster, *C. gigas*, larvae were cultured at the South Australian Research and Development Institute in Adelaide, South Australia with larvae derived from nineteen families, reared in 1 µm filtered seawater (FSW) at 24.5 ± 0.5 °C with a salinity of 36.5 ± 0.5 ppt as previous described (107). Larvae were fed daily with an algal mixture of *Tisochrysis lutea*, *Pavlova lutheri*, *Chaetoceros muelleri*. and *Chaetoceros calcitrans*.

At 17 days post fertilisation (dpf) and with a shell length of 300–330 µm, approx. 250 larvae were exposed to epinephrine hydrochloride (Sigma-Aldrich) at a concentration of 10^{-4} M in a total volume of 2.5 ml FSW in glass shell vials (outside diameter x height: 29 × 94 mm). Exposure was terminated after 3 h by removing the EPI-containing seawater and adding 10 ml fresh FSW with algal feed. Larvae treated similarly, but without epinephrine exposure, were used as controls. After 24 h, metamorphosed individuals were assessed for the three replicates per treatment using an inverted microscope by counting live larvae, dead larvae and spat (juveniles with clear adult shell growth). No significant mortality was recorded for any biological replicate of the assay. Metamorphosis success was calculated as a percentage of larvae that successfully completed metamorphosis, with significant differences observed between EPI-treated and untreated individuals as assessed using a t-test in R v4.02 with significance at a probability level of 0.05.

Gene expression analysis

The gene expression of 6 *C. gigas* caspases were evaluated in oyster larvae during late larval development (14 dpf, 15 dpf, 16 dpf), prior to metamorphosis induction (17 dpf), 3 hours and 6 hours post exposure start (hpe) to a 3 h epinephrine exposure (E3 and E6), as well as in spat 24 hpe. For each

sample point, three biological replicates were preserved in PaxGene Tissue system (PreAnalytix) and stored at -20 °C.

Primers for Cg2A, Cg8B, Cg3/7, Cg3A, Cg3B, and Cg3C were designed with Primer Blast at NCBI (108) with an amplicon length ranging from 150–196 base pairs (bp). The elongation factor-1 α , ribosomal protein S18, ribosomal protein L7 were chosen as reference genes as previously described (107, 109). Primer pairs were optimised for final concentrations, annealing temperatures and MgCl₂ concentrations (Additional file 6); their specificities were verified by sequencing. Total RNA was extracted from all biological replicates per sample points (~ 20–40 mg per sample) using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich) following the manufacturer's protocol. Genomic DNA was removed using RQ1 RNase-Free DNase (Promega). One μ g of clean total RNA was reverse-transcribed to cDNA using oligo(dT)18 primers with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) in 10 ml reaction volume with 0.5 μ l cDNA was used for the quantitative PCR (qPCR) reactions, which were run in duplicates on a 384 well plate PCR thermal cycler Light Cycler 480 Instrument II (Roche). The qPCR conditions were as follows: 95 °C for 10 min, 45 cycles of 95 °C for 15 sec, 60–62 °C (primer pair dependent) for 30 sec and 72 °C for 30 sec. A melt curve was run at the end at 65–95 °C with a temperature transition rate of 0.05 °C. A non-template control and a cDNA dilution series for each primer pair were analysed in parallel to assess primer efficiency (standard curve) and exclude contamination. Primer efficiency and relative gene expression were based on a modified comparative Ct model as described in (110). The average relative gene expression and standard error for each sample per caspase primer were calculated and significant differences between each sample point were assessed using a one-way ANOVA followed by multiple pairwise comparisons using a Tukey's Honestly Significant Difference Test in R v4.02 with significance at a probability level of 0.05.

Abbreviations

AIF: apoptosis inducing factors, Apaf-1: apoptotic protease activating factor-1, ASC: apoptosis-associated speck-like proteins, BI: Bayesian Inference, CARD: caspase-recruitment domain, CASc: caspase-specific domain, Cyt c: cytochrome c, DAMPs: danger-associated molecular patterns, DD: death domains, DED: death-effector domain, DISC: death-inducing signalling complex, dpf: days post fertilisation, DSRM: double stranded RNA-binding domains, EPI: epinephrine, EST: expressed sequence tags, FADD: Fas-associated protein with death domain, FSW: filtered seawater, hpe: hours post exposure start, H-R: hypoxia-reoxygenation, IAP: Inhibitors of apoptosis, IL: interleukins, LPS: lipopolysaccharides, ML: Maximum Likelihood, MOMP: mitochondrial outer membrane, NCBI: National Center for Biotechnology Information, NLRPs: cytoplasmic Nod-like receptors, PAMPs: pathogen-associated molecular patterns, PARP: poly ADP-ribose polymerase, PIDD: p53-induced protein with death domain, RAIDD: RIP-associated Ich-1/Ced-3-homologue protein with a death domain, RGN: regucalcin, ROS: reactive oxygen species, TLR: toll-like receptors,

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Data availability

The datasets used and/or analysed during the current study are mostly available in the Additional files. Additional datasets are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

Experimental design and conceptualization were generated by SV and AJ. All laboratory studies, phylogenetic calculations and BLAST searches were completed by SV. Interpretation of data and manuscript preparation were conducted by SV, AJ, SC XL.

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Additional Files

Additional File 1: Alignments of full protein (A) and coding sequence CDS (B) sequences of *Crassostrea gigas* caspase homologues: caspase-1 (27), caspase-1 (28), caspase-3 (29) and Cg3B (identified in this study).

Additional File 2: Accession numbers and additional information about caspase protein sequences used for phylogenetic analysis of initiator and executioner caspase.

Additional File 3: Alignment of CASc domain of initiator caspases. Light blue coloured: used alignment for phylogenetic analyses. Orange underscore: large p20 subunit, blue underscore: small p10 subunit, black underscore: intersubunit linker.

Additional File 4: Alignment of CASc domain of executioner caspases. Light blue coloured: used alignment for phylogenetic analyses. Orange underscore: large p20 subunit, blue underscore: small p10 subunit, black underscore: intersubunit linker.

Additional File 5: Alignment of CARD domains of initiator caspases.

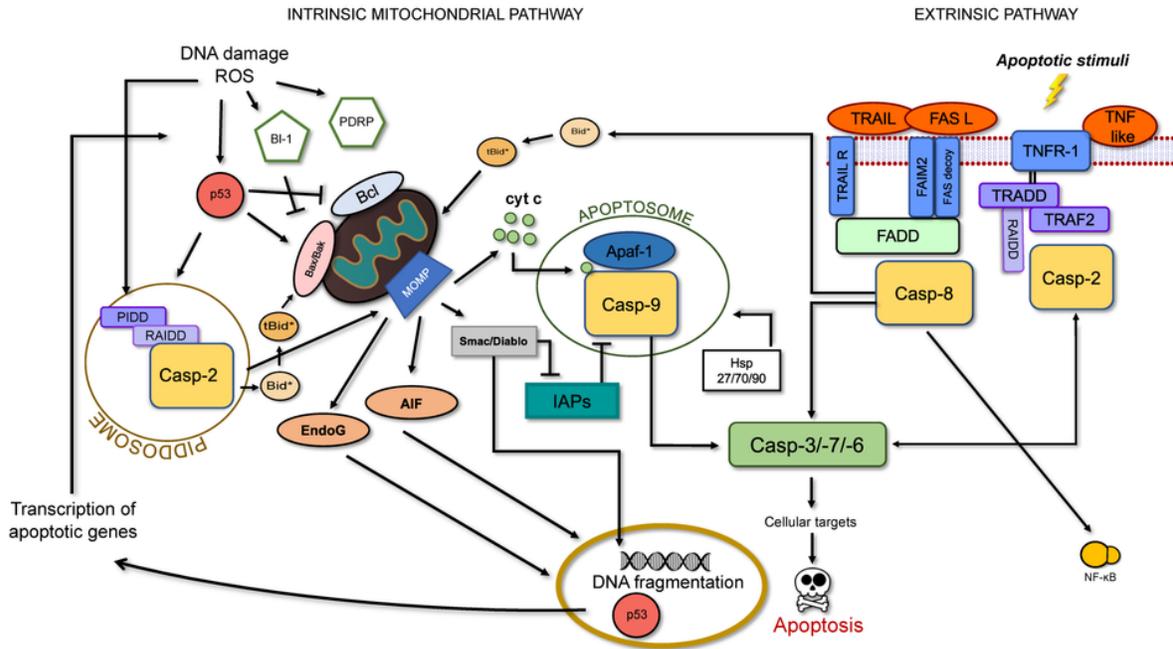
Additional File 6: Alignment of DED-DED domains of initiator caspases. Green underscored: DED domains.

Additional File 7: Primers for quantitative gene expression analysis of caspases in *Crassostrea gigas*. EF1: elongation factor 1, RS18: ribosomal protein S18, RL7: ribosomal protein L7, Fwd: forward primer; Rev: reverse primer.

Figures

APOPTOSIS

Bivalves



Drosophila melanogaster

Caenorhabditis elegans

PYROPTOSIS- Inflammation

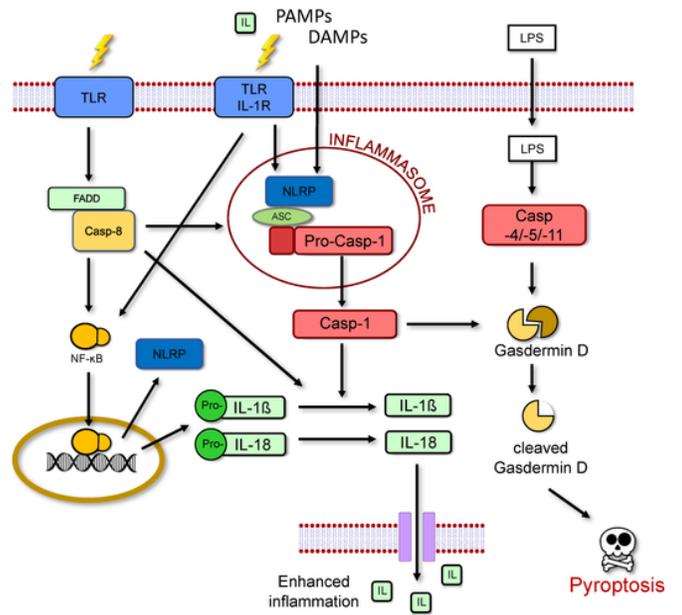
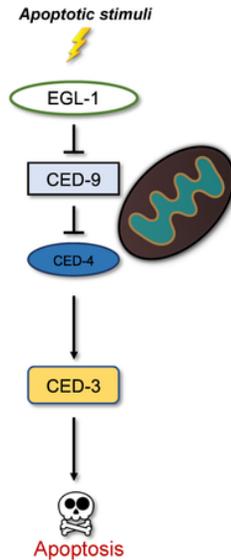
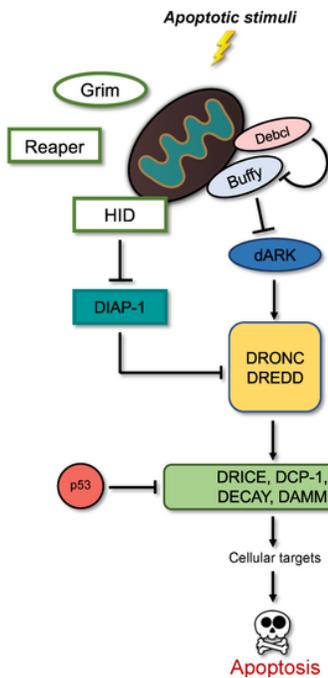


Figure 1

Schematic representation of A) potential apoptotic pathways in bivalve species based on homologous genes characterised in bivalves or suggested in bivalve genomes (*not identified in a bivalve species yet) to the vertebrate's intrinsic mitochondrial or extrinsic apoptotic pathways as well as apoptotic pathways in *Drosophila melanogaster* and *Caenorhabditis elegans*. B) Pyroptotic pathways in vertebrates. (Adopted from (6, 7, 9, 17, 19, 20, 27, 48, 111, 112))

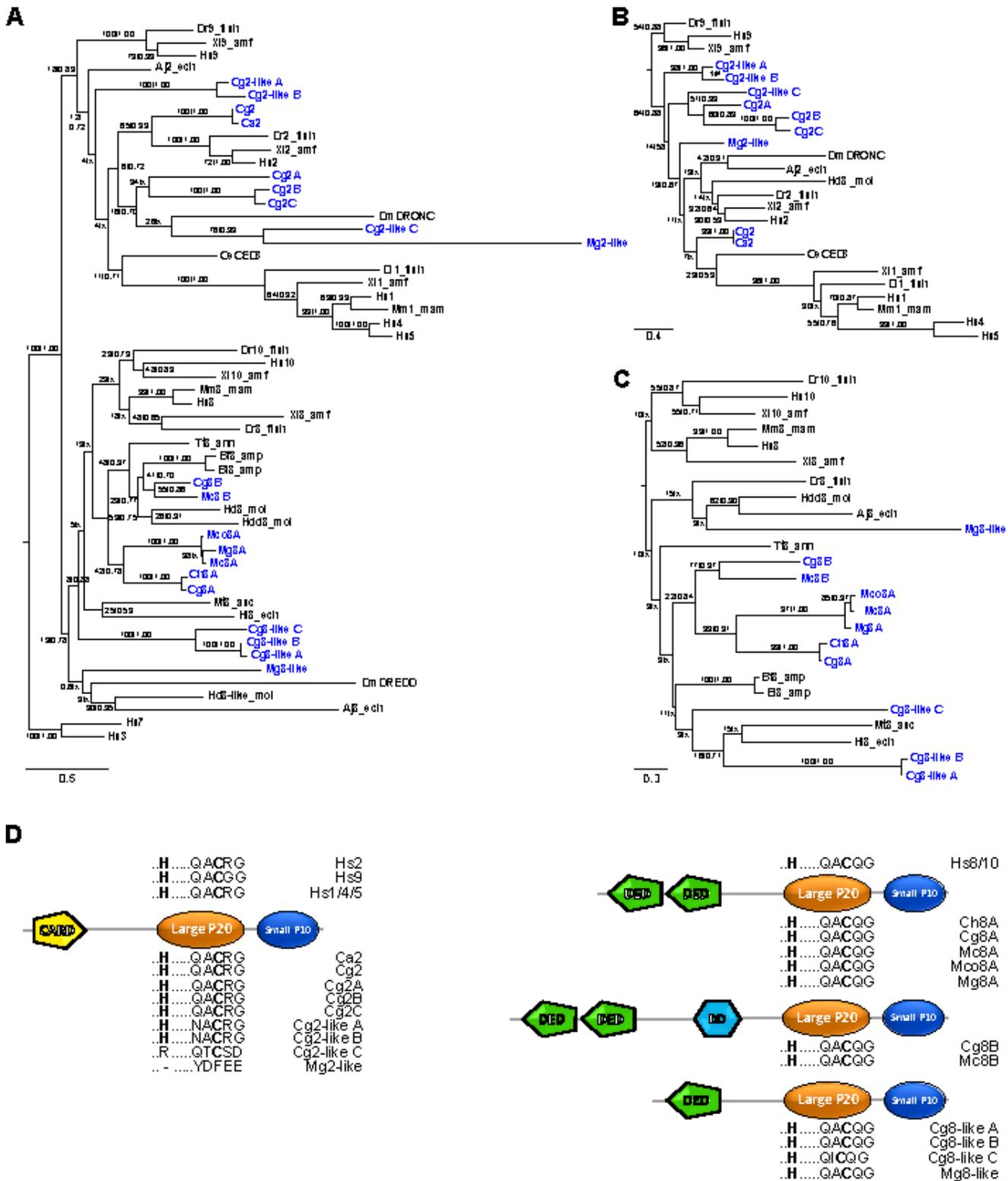


Figure 2

Phylogenetic relationship of initiator caspases in bivalves (blue) compared to other vertebrate and invertebrate homologs (black). A) Phylogenetic tree of trimmed large and small caspase domains of all initiator caspases. Values above/below nodes separated by slash show bootstrap support values for Maximum Likelihood analysis as percentage of bootstrap values and the Bayesian Inference posterior probabilities. Human caspase-3 and caspase-7 homologs used as outgroup. B & C) Phylogenetic

relationship of caspase-recruitment domain (CARD) (B) or single/double death-effector domains (DED) (C). D) Schematic representation of initiator caspase structure of bivalves with the CARD, DED or death domain (DD) motifs in their prodomains and the two caspase specific domains: large p20 and small p10 domain. The p20 active sites motif (..H.....QACXG) with the conserved histidine and cysteine residue in bold is shown for each bivalve caspase homolog. Aj: *Apostichopus japonicus*, Bf: *Branchiostoma floridae*, Bl: *Branchiostoma lanceolatum*, Ca: *Crassostrea angulata*, Ce: *Caenorhabditis elegans*, Cg: *Crassostrea gigas*, Ch: *Crassostrea hongkongensis*, Dl: *Dicentrarchus labrax*, Dm: *Drosophila melanogaster*, Dr: *Danio rerio*, Hd: *Haliotis diversicolor*, Hdd: *Haliotis discus discus*, Hl: *Holothuria leucospilota*, Hs: *Homo sapiens*, Mc: *Mytilus californianus*, Mco: *Mytilus coruscus*, Mg: *Mytilus galloprovincialis*, Mm: *Mus musculus*, Mt: *Molgula tectiformis*, Tt: *Tubifex tubifex*, Xl: *Xenopus laevis*. _amf: amphibian, -amp: amphioxus, _ann: annelid, _asc: ascidian, _ech: echinoderm, _fish: fish, _mam: mammal, _mol: mollusc.

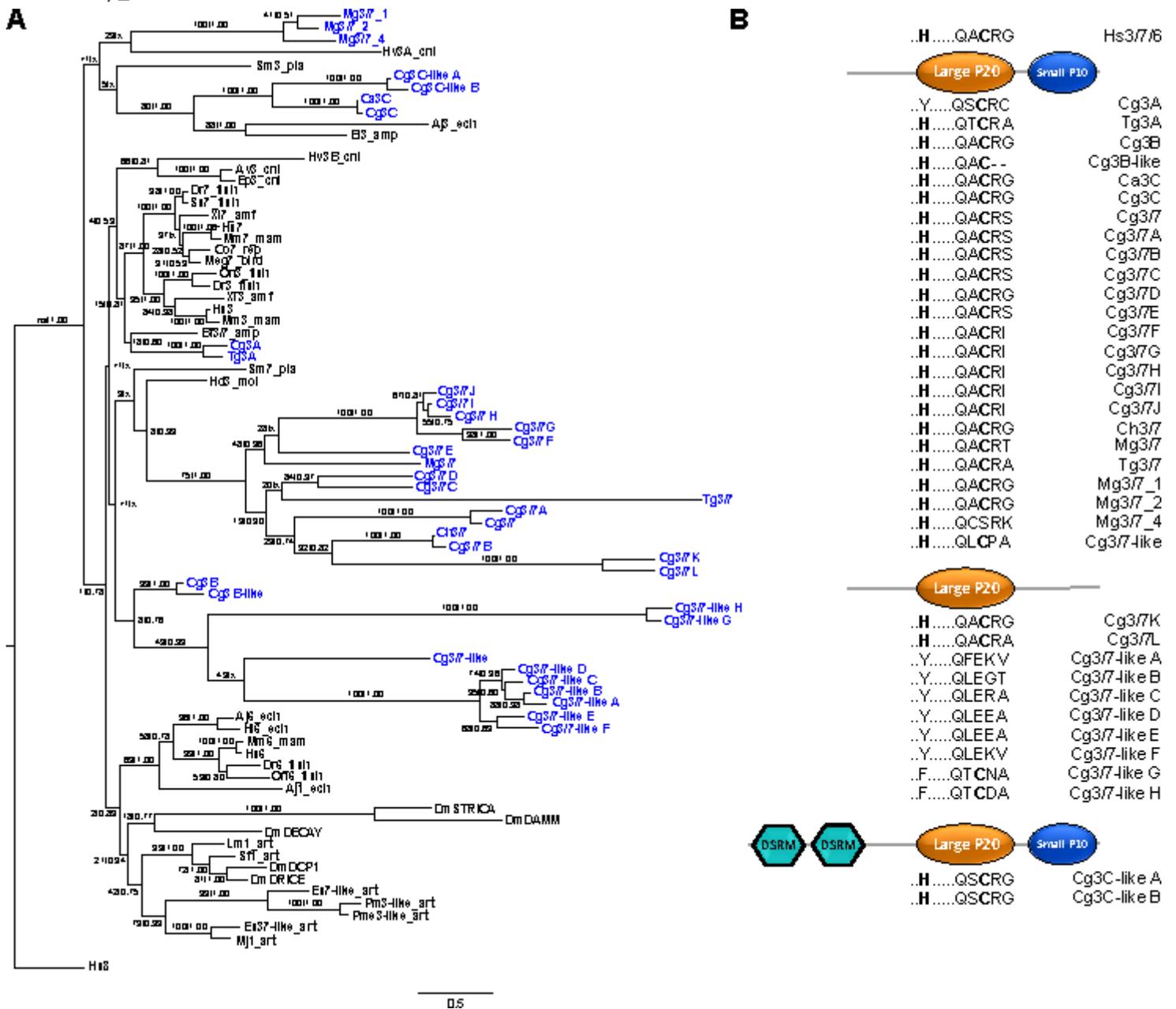


Figure 3

Phylogenetic relationship of executioner caspases in bivalves (blue) compared to other vertebrate and invertebrate homologs (black). A) Phylogenetic tree of trimmed large and small caspase domains of all executioner caspases. Values above/below branches separated by slash show bootstrap support values for Maximum Likelihood analysis as percentage of bootstrap values and the Bayesian Inference posterior probabilities. Human caspase-8 used as outgroup. na: not available. B) Schematic representation of caspase structure with two caspase specific domains: large p20 and small p10 domain. The p20 active sites motif (.H.....QACXG) with the conserved histidine and cysteine residue in bold is shown for each bivalve caspase homolog. DSRM: double stranded RNA-binding motif, a unique prodomain in the bivalve Cg3C-like A and Cg3C-like B. Aj: *Apostichopus japonicus*, Av: *Anemonia viridis*, Bl: *Branchiostoma lanceolatum*, Bf: *Branchiostoma floridae*, Ca: *Crassostrea angulata*, Cg: *Crassostrea gigas*, Ch: *Crassostrea hongkongensis*, Co: *Cynops orientalis*, Dm: *Drosophila melanogaster*, Dr: *Danio rerio*, Ep: *Exaiptasia pallida*, Es: *Eriocheir sinensis*, Hd: *Haliotis diversicolor*, Hl: *Holothuria leucospilota*, Hs: *Homo sapiens*, Hv: *Hydra vulgaris*, Lm: *Locusta migratoria*, Meg: *Meleagris gallopavo*, Mg: *Mytilus galloprovincialis*, Mj: *Marsupenaeus japonicas*, Mm: *Mus musculus*, On: *Oreochromis niloticu*, Pm: *Penaeus monodon*, Pme: *Penaeus merguensis* Sf: *Spodoptera frugiperda*, Sm: *Schistosoma mansoni*, Ss: *Salmo salar*, Tg: *Tegillarca granosa*, Xl: *Xenopus laevis*. *_amf*: amphibian, *_amp*: amphioxus, *_art*: arthropod, *_bird*: bird, *_cni*: cnidarian, *_ech*: echinoderm, *_fish*: fish, *_mam*: mammal, *_mol*: mollusc, *_pla*: plathelminth, *_rep*: reptile.

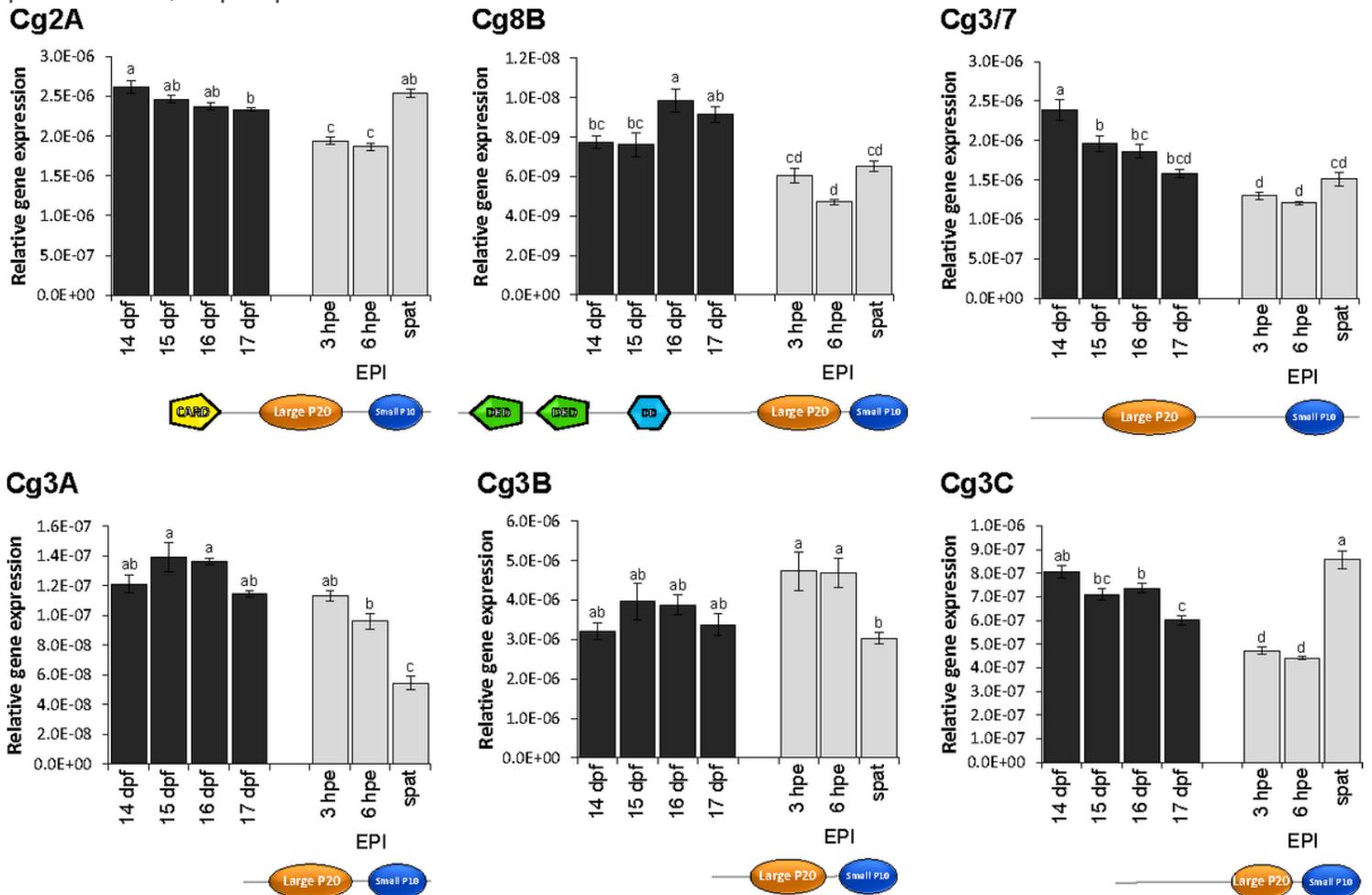


Figure 4

Relative gene expression of two initiator caspases, Cg2A and Cg8B, and four executioner caspases, Cg3/7, Cg3A, Cg3B, Cg3C, during Pacific oyster late larval development (14-17 days post fertilisation (dpf)), after 3 and 6 hours post exposure start (3 hpe & 6 hpe) to a 3 h epinephrine (EPI) exposure at 10⁻⁴ M for metamorphosis induction, and in spat (24 hpe). Each gene expression profile is accompanied by a schematic representation of the key domains present in the protein sequence of the analysed caspase. Different lower-case letters represent significant differences with p<0.05. CARD: caspase-recruitment domain, DED: death-effector domain, DD: death domain, Large p20: large caspase subunit p20, Small p10: small caspase subunit p10.

Supplementary Files

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