Coping with antagonistic predation risks: Predator-dependent unique responses are dominant in Ceriodaphnia cornuta

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Abstract

Inducible defenses of prey are evolved under diverse and variable predation risks. However, during the co-evolution of prey and multiple predators, the responses of prey to antagonistic predation risks, which may put the prey into a dilemma of responding to predators, remain unclear. Based on antagonistic predation pressure from an invertebrate (Chaoborus larvae) and a vertebrate (Rhodeus ocellatus) predator, we studied the responses of multiple traits and transcriptomes of the freshwater crustacean Ceriodaphnia cornuta under multiple predation risks. Chaoborus predation risk altered the expression of genes encoding cuticle proteins and modulated the biosynthesis of steroid hormones, cutin, suberine, and wax, leading to the development of horns and increase in size at the late developmental stage. Meanwhile, fish predation risk primarily triggered genes encoding ribosomes and those involved in unsaturated fatty acid biosynthesis and cysteine and methionine metabolism, resulting in smaller individual size and earlier reproduction. Inducible responses of both transcriptome and individual traits revealed that predator-dependent unique responses were dominant and the dilemma of antagonistic responses was relatively limited. However, the unique individual traits in response to invertebrate predation could be significantly impaired by vertebrate predation risk, even though the unique responses to different predators were extremely weakly correlated and could be elicited simultaneously. These results indicate that diverse predator-dependent unique responses are favored by Ceriodaphnia during its co-evolution with multiple predators. Nonetheless, Ceriodaphnia is not a generalist that can fully adopt all predator-dependent unique responses simultaneously under multiple predation risks.

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Running title: Inducible responses to multiple predators

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Abstract

Inducible defenses of prey are evolved under diverse and variable predation risks. However, during the co-evolution of prey and multiple predators, the responses of prey to antagonistic predation risks, which may put the prey into a dilemma of responding to predators, remain unclear. Based on antagonistic predation pressure from an invertebrate (*Chaoborus* larvae) and a vertebrate (*Rhodeus ocellatus*) predator, we studied the responses of multiple traits and transcriptomes of the freshwater crustacean *Ceriodaphnia cornuta* under multiple predation risks. *Chaoborus* predation risk altered the expression of genes encoding cuticle proteins and modulated the biosynthesis of steroid hormones, cutin, suberine, and wax, leading to the development of horns and increase in size at the late developmental stage. Meanwhile, fish predation risk primarily triggered genes encoding ribosomes and those involved in unsaturated fatty acid biosynthesis and cysteine and methionine metabolism, resulting in smaller individual size and earlier reproduction. Inducible responses of both transcriptome and individual traits revealed that predator-dependent unique responses were dominant and the dilemma of antagonistic responses was relatively limited. However, the unique individual traits in response to invertebrate predation could be significantly impaired by vertebrate predation risk, even though the unique responses to different predators were extremely weakly correlated and could be elicited simultaneously. These results indicate that diverse predator-dependent unique responses are favored by *Ceriodaphnia* during its co-evolution with multiple predators. Nonetheless, *Ceriodaphnia* is not a generalist that can fully adopt all predator-dependent unique responses simultaneously under multiple predation risks.

Keywords: Cladoceran; *Chaoborus* ; Fish; Inducible defense; Predation risk

Introduction

In the co-evolution of predators and prey, defense is critical for prey survival. Under variable predation risks, inducible defenses triggered by predation cues are favored by prey (Tollrian & Harvell, 1999). For successful defense against predators, prey organisms adopt various inducible protective characteristics, including behavioral (De Meester, 1993), morphological (Gu et al., 2021), chemical defense (Selander et al., 2015), and life history (Kvile, Altin, Thommesen, & Titelman, 2021) traits. Since diverse predation risks can prevent the stable expression of an inducible defensive trait (Steiner & Auld, 2012), the present study sought to understand the responses of prey to multiple predation risks, particularly to predators exerting antagonistic selection pressures.

Inducible defenses are common in aquatic organisms, such as phytoplankton (Lürling, 2020), zooplankton (Diel, Kiene, Martin-Creuzburg, & Laforsch, 2020), amphibians (Mitchell, Bairos-Novak, & Ferrari, 2017), and fish (Brönmark & Miner, 1992). Through the integration of inducible defense research, we classified responses to multiple predators into two major types. The first type is the general response, which evolves through diffusion co-evolution and represents reciprocal adaptation in response to similar predators; for instance, mayflies adopt the same avoidance behavior under predation risks from different fish (Alvarez, Landeira-Dabarca, & Peckarsky, 2014). The second type is the specific response, which evolves through pairwise co-evolution between specific predators and prey, such as the immune responses to pathogens (Westra et al., 2015) and the inducible crests of *Daphnia* in response to *Notonecta* predation (Grant & Bayly, 1981). Moreover, the specific responses of different traits can be further subdivided into antagonistic responses to the same trait and unique responses to separate traits. Under antagonistic selection pressures, if unique responses to separate traits are dominant, the complex defense responses may incur maintenance cost, that is, energetic costs of the sensory and regulatory mechanisms. Moreover, if the prey primarily exhibits antagonistic responses of the same traits, inducible responses to a predator may incur an environmental cost, that is, vulnerability to other predators (Auld, Agrawal, & Relyea, 2010); this environmental cost is further linked to selection and plays a dual role in the evolution of inducible defenses (Decaestecker, De Meester, & Ebert, 2002). Therefore, we hypothesized that predator-dependent unique responses are dominant, helping, at least in part, avoid the dilemma of the prey for responding to predators under multiple predation risks.

In aquatic ecosystems, cladocerans are at the middle of the food chain, acting as a food resource for insects

and fishes (Miner, De Meester, Pfrender, Lampert, & Hairston, 2012). These invertebrate and vertebrate predators constitute antagonistic selection pressures on the size or habitat selection of waterfleas; for instance, larger plankton are vulnerable to large visual predators (e.g., fish) but less vulnerable to small ambush predators (e.g., *Chaoborus* larvae) (Swift, 1992). Presumably, inducible responses of *Daphnia* to visual predators primarily include life history changes (Effertz & Von Elert, 2014), which are rather different from the defensive traits triggered by invertebrate predators, such as the development of "twist" (Herzog, Rabus, Ribeiro, & Laforsch, 2016), neck teeth (Tollrian, 1993), and horns (Gu, Qin, Zhu, et al., 2020). These reports support our hypothesis.

Antagonistic inducible traits are commonly expressed by *Daphnia* . For instance, *Daphnia hyalina* shows completely opposite responses of size and reproduction under predation pressures by vertebrates and invertebrates (Stibor & Lüning, 1994). *Daphnia galeata* prefers deeper habitats under fish predation risk, while inhabits upper water layers under *Chaoborus* predation risk (Dodson, 1988). In addition, general responses, such as the development of an elongated tail spine, are observed in *Daphnia* in response to fish, *Triops* , and *Notonecta* (Gu, Qin, Lu, et al., 2020; Ritschar, Rabus, & Laforsch, 2020). Consistent with inducible defensive traits, both general and specific responses appear at the molecular level; for instance, in *Daphnia magna* , actin and tubulin expression is decreased under *Chaoborus* larvae or fish predation risks (Pijanowska & Kloc, 2004), ribosomal protein and vitellogenin expression is increased under fish predation risk (Effertz, Mueller, & Von Elert, 2015), and cuticle protein expression is increased but vitellogenin expression is decreased under *Triops* predation risk (Otte, Fröhlich, Arnold, & Laforsch, 2014). Overall, in *Daphnia* , a given species exhibits various types of responses under antagonistic predation risks. However, from these sporadic studies on different *Daphnia* species and clones, we cannot determine the precise type of response preferred by the prey.

Ceriodaphnia cornuta is a widely distributed species with sensitive inducible defensive traits (Gu et al., 2021; Qin et al., 2021), providing a suitable model for testing our hypothesis. Since some inducible traits are hidden (Laforsch, Ngwa, Grill, & Tollrian, 2004), research on a few traits is insufficient. In recent years, omics technologies have furthered our understanding of the mechanisms of inducible defense (Hales et al., 2017; Zhang et al., 2021). Therefore, to test our hypothesis that predator-dependent unique responses are dominant, we assessed multiple traits and transcriptomes of *C. cornuta* in response to predation pressures from *Chaoborus* larvae and fish, respectively. Additionally, to test the hypothesis that predator-dependent unique responses help prey avoid the dilemma of responding to predators, we examined the expression of individual traits under joint predation risks and explored the correlations between various inducible responses.

Materials and methods

Predation risks

Predation risks were simulated using different predator-conditioned medium prepared following the methodology described by Gu, Qin, Zhu, et al. (2020). We cultured four *Rhodeus ocellatus* or 100 *Chaoborus* sp. larvae in aged tap water, fed them *C. cornuta* for 6 h, and then transferred them into 2 L of COMBO medium (Kilham, Kreeger, Lynn, Goulden, & Herrera, 1998) for 18 h. The predator-conditioned medium stock containing different predator kairomones (Hahn, Effertz, Bigler, & von Elert, 2019; Weiss et al., 2018) was passed through a 0.22 μm glass fiber filter (Millipore), and the filtrate was stored in a refrigerator until experiments. The control (C) included COMBO medium. The fish (F) and *Chaoborus* (CH) predation risk treatments included respectively 20- (i.e., 1 fish per 10 L) and 2.5-fold (i.e., 20 *Chaoborus* larvae per liter) diluted filtered medium stocks. Finally, the combination treatment (CH + F), representing joint predation risks, included both diluted medium stocks.

Life history experiment

The *C. cornuta* clone used in this experiment was sampled from Lake Taihu (31°22'13.548"N, 120deg0'16"E), China. *C. cornuta* was cultured in COMBO medium at 25degC and 500 lx fluorescent light intensity under a

14:10 h light/dark cycle and fed *Chlorella pyrenoidosa*(1.5 mg C^*L^{-1}). Synchronous *C. cornuta* at a density of 1 individual per 10 mL, was adapted to the above conditions for at least two generations. To test the type of inducible responses of individual traits as well as the response strategy under joint predation risks, we set up a full factor experiment containing C, F, CH, and CH + F. We randomly divided newborn individuals into different treatments within 12 h. Each individual was cultured in 10 mL of medium, with 10 replicates per treatment. The media in different treatments were refreshed daily. Experimental workflow is presented in Figure S1.

Body size and horns were measured at maturity and at the late developmental stage (i.e., at 16th day). Horns were scored following the method described by Gu et al. (2021): absent (score 0), small (score 5), and large (score 10). Individual scores were normalized by a maximum score to define the induction level (0%–100%). In addition, time to the first brood, neonate size, brood number, total offspring number, and average brood size were recorded.

RNA extraction and sequencing

To accurately evaluate the type of response at the transcriptional level, we sequenced the transcriptomes of *C. cornuta* under C, F, and CH treatments. Groups of 250 newborn individuals were cultured in 2.5 L of medium with three replicates per treatment. During cultivation, responses triggered by different predation risks were verified through inducible traits, that is, horns and body size at maturity. The medium was refreshed daily, and samples were collected within 12 h after the first brood. The samples were frozen in liquid nitrogen and homogenized in TransZol Up. Total RNA was extracted using the TransZol Up Plus RNA Kit (ER501, TRANS, China), following the manufacturer's instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and verified using agarose gel electrophoresis. The RNA integrity number of all samples was > 7.0.

The total mRNA of *Ceriodaphnia* was enriched by Oligo (dT) beads. The enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse-transcribed into cDNA using random primers. The cDNA fragments were purified, end-repaired, poly(A)-added, and ligated to Illumina sequencing adapters. The ligation products were size-selected and PCR-amplified to develop a cDNA library. The cDNA library was sequenced using Illumina HiSeq^{TM} 4000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Transcript assembly and annotation

Since genomic sequencing of *Ceriodaphnia* has not been conducted to date, we adopted *de novo* RNA-Seq to analyze the *C. cornuta* transcriptome. To obtain high-quality clean reads, sequenced reads were cleaned by removing reads containing adapters, $> 10\%$ unknown nucleotides (N), and low-quality reads (Q-value [?] 20). Clean reads were assembled into unigenes using Trinity (Grabherr et al., 2011). To annotate the unigenes, we used the BLASTx program with an E-value $< 10^{-5}$ in the NCBI non-redundant protein (Nr), SWISS-PROT protein, Kyoto Encyclopedia of Genes and Genomes (KEGG), and COG/KOG databases. Functional protein annotations were obtained according to the best alignment results.

Gene expression analysis and RT-qPCR

Unigene expression was quantified and normalized to reads per kb per million (RPKM) (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008). Differential transcript expression analysis between the control and different predation risk treatments was performed using DESeq2. Genes with a false discovery rate (FDR) < 0.05 and absolute fold change [?] 1 were considered differentially expressed genes (DEGs). We categorized the DEGs into different types according to our classification and then used KEGG pathways for functional analysis. Pathways with P [?] 0.05 were considered significantly enriched. The representative DEGs of various significantly enriched pathways were selected according to the following precedence conditions: stable expression, large fold change, and pathways annotated in a closely related species, that is, *D. magna* and *Daphnia pulex* .

To validate the RNA-Seq data in the *C. cornuta* transcriptome, we quantified the expression of 18 random DEGs using RT-qPCR. ddH_2O was used as the negative control. cDNA was synthesized from mRNA using Reverse Transcriptase SuperMix (R233, Vazyme, China), and RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Q711, Vazyme, China). All primer sequences are listed in Table S1. We obtained expression data for four alternative reference genes (Scoville & Pfrender, 2010) and calculated their average gene expression stability using geNorm. Glyceraldehyde-3-phosphate dehydrogenase (G3PD), RNA polymerase II (RNAP II), and elongation factor 1-alpha genes (EF) were determined to be stably expressed and geometrically averaged to calculate the gene expression normalization factor for each sample. Gene expression was quantified using the $2^{-\Delta\Delta\tau}$ method. The concordance between RNA-Seq and RT-qPCR data was assessed using regression analysis.

Statistical analysis

To test the effects of different predation risks, a MANOVA was performed on individual traits. When the residuals were normally distributed (Shapiro–Wilk test) and variances were homogeneous (Levene's test), the data for each trait were evaluated by two-way ANOVA, followed by Bonferroni comparisons between different groups. When the normality test failed, the Scheirer–Ray–Hare test was used, followed by the Wilcoxon rank-sum test to assess significant differences among the different treatments. In the present study, statistical significance was set at $P < 0.05$. To simplify the effects of joint predation risks on different types of responses, we considered our results based on the conceptual diagram of possible prey responses in the combined predator treatment (Fig. S2). To test the correlations between different types of responses, we determined Pearson's correlation coefficients between traits as well as between representative DEGs in different categories. The above statistical tests were performed in R (version 3.6.2). In addition, correlation, principal component, DEGs, and pathway enrichment analyses were conducted using OmicShare Tools (*https://www.omicshare.com/tools/*).

Results

Morphology and life history traits

The different predation risks significantly triggered various responses of morphology and life history traits (Table 1). Compared with the control, the responses induced by fish and *Chaoborus* predation risks could be classified into the following four categories (Fig. 1). (1) Unique responses to *Chaoborus* larvae [horn expression (at maturity and 16th day) and total offspring number]: *C. cornuta*developed horns (maturity: $P < 0.001$; 16th day:$P < 0.001$) and produced more offspring ($P = 0.002$) under *Chaoborus* predation risk, although these responses were not significant under fish predation risk. (2) Unique responses to fish (time to first brood and neonate size): the neonate size ($P= 0.006$) and time to first brood ($P < 0.001$) of*C. cornuta* were significantly decreased under fish predation risk, although these responses were not significant under*Chaoborus* predation risk. (3) General responses (size at maturity and brood number): the size (CH vs. C: $P = 0.007$; F vs. C:$P < 0.001$) and brood number (CH vs. C: $P = 0.006$; F vs. C: $P = 0.017$) were significantly decreased under both fish and *Chaoborus* predation risks. (4) Antagonistic responses (size at 16th day): *C. cornuta* size increased under*Chaoborus* predation risk ($P = 0.034$) but decreased under fish predation risk ($P < 0.001$). Additionally, no significant differences were observed in average brood size. Thus, the unique responses in individual traits of *C. cornuta* to different predators were dominant: unique responses (five traits) > general responses (two traits) > antagonistic responses (one trait).

Inducible responses under joint predation risks

Antagonistic responses would put the prey into a dilemma regarding which predator to respond to (Fig. S2). In our experiments, size at 16th day was not significantly expressed under joint predation risks (CH + F vs. C: $P = 0.764$). Consistently, unique responses to *Chaoborus*predation risk were significantly affected by the presence of fish predation risk, that is, horn expression in the combination treatment was greatly impaired compared with that under the *Chaoborus*predation risk (maturity: $P = 0.012$; 16th day: $P<$

0.001). This effect was highly correlated to individual development, that is, in the combination treatment, *C. cornuta*horns were significantly expressed at maturity (CH + F vs. C: $P = 0.007$) but not at 16th day. Conversely, the effects of joint predation risks were observed in neither unique responses to fish nor general responses. As such, the responses of size at maturity (CH + F vs. C:$P < 0.001$), time to first brood (CH + F vs. C: $P= 0.027$), and neonate size (CH + F vs. C: $P = 0.003$) were significantly altered in the combination treatment but were not significantly different from those under the fish predation risk treatment. Thus, various predator-dependent unique responses could be simultaneously expressed under antagonistic predation risks, although the prey could not completely avoid the effect of other predation risks, and the co-expression of unique responses was related to individual development. These results basically support our hypotheses.

Overview of the assembled transcriptome

We obtained respectively 78,341,484, 72,493,072, and 69,930,191 clean reads for C, CH, and F. A total of 37,120 unigenes were assembled using Trinity, and each sample contained > 66.54% assembled unigenes (Table S2). Through comparisons against the Nr, KEGG, COG, and SWISS-PROT databases, 18,343 unigenes (49.4%) were annotated (Fig. S3). Regarding species distribution in the Nr database, *C. cornuta*showed the highest comparison rate with *D. magna* (26.33%), followed by *D. pulex* (2.6%) (Fig. S4A). The unigenes enriched in the KOG database were classified into transcription, ribosomal structure, gene replication, recombination, and repair (Fig. S4B). The annotated GO terms were mainly associated with metabolic processes, cellular processes, cell parts, and binding (Fig. S4C).

DEGs

Paired samples within the same treatment showed high Pearson's correlation coefficients ([?]0.90) and were clustered in principal component analysis (Fig. 2), which conformed to the requirements of biological repetition. Furthermore, the expression patterns of DEGs showed significant concordance between RT-qPCR and RNA-Seq (Fig. S5), indicating that our RNA-Seq analysis of the expression data was reliable.

Compared with the control, *Chaoborus* and fish predation risks significantly affected the expression of 1,515 and 846 genes, respectively (Fig. 3A). Among these, there were 1,399 unique DEGs in CH, 730 unique DEGs in F, 114 general DEGs, and two antagonistic DEGs (Fig. 3B and Table S3). Considering the DEGs triggered by different predators, we further analyzed the differences in the enriched pathways of *C. cornuta* (Supplementary Table S4) and identified the major DEGs and pathways related to inducible defensive traits (Table 2). The DEGs of*C. cornuta* under *Chaoborus* predation risk, including cuticle protein, fatty acyl-CoA reductase, and trypsin genes, were mainly enriched in cutin, suberine, and wax biosynthesis; protein digestion and absorption; and steroid hormone biosynthesis. Meanwhile, the DEGs of *C. cornuta* under fish predation risk, including ribosomal protein, actin, and short-chain type dehydrogenase genes, were mainly enriched in cysteine and methionine metabolism, ribosome, phototransduction, and unsaturated fatty acid biosynthesis. The general DEGs, including cysteine proteinase, HSP70, actin, and alpha-tubulin genes, were enriched in apoptosis pathways and antigen processing and presentation. Therefore, specific unique responses to different predators were dominant at the transcriptional level: unique DEGs (2,129 genes) > general DEGs (114 genes) > antagonistic DEGs (2 genes).

Correlation between different inducible responses

Nine individual traits and 40 representative DEGs (Table S5) revealed strong Pearson's correlations within unique responses to fish or*Chaoborus* larvae (Fig. 4). For instance, the response of horn expression at maturity was significantly correlated with changes in horns at 16th day ($P = 0.05$) and total offspring number ($P= 0.05$). However, the unique responses to fish and *Chaoborus*larvae were weakly correlated in both analyses. Only a few significant correlations appeared on the weak correlation area in transcriptional expression. For instance, Unigene0008297 in ribonucleoprotein component protein expression was significantly and positively correlated with Unigene0027903 ($P= 0.049$) and Unigene0005204 ($P= 0.013$) in aspartokinase and adeno-

sylhomocysteinase expression, respectively (Fig. 4). Therefore, the unique responses triggered by different predators are weakly coupled.

Discussion

Our experiments proved that *C. cornuta* responds differently to antagonistic predation risks of fish and *Chaoborus* larvae. Based on the classification of inducible responses, our results revealed for the first time that specific predator-dependent unique responses are dominant, followed by general responses, whereas the antagonistic responses are rare. In addition, the unique responses triggered by different predators were extremely weakly coupled and could be elicited simultaneously. Overall, the experimental results support our hypothesis that predator-dependent unique responses are dominant, helping the prey avoid the dilemma of which predator to respond to. Nonetheless, the unique responses to *Chaoborus* larvae cannot completely avoid the effects of fish predation risk, implying the presence of complex costs and limitations of unique responses to multiple predators.

In *C. cornuta* , horn expression and body enlargement are adaptive inducible traits to *Chaoborus* larva predation (Gu et al., 2021; Riessen & Trevett-Smith, 2009). A larger individual size at late developmental stages requires rapid growth and high food intake (Gianuca, Pantel, & De Meester, 2016), thereby promoting the brood and total offspring numbers. The horns are formed by the carapace, which comprises two layers of dermal cells and is covered by chitin in combination with cuticle proteins (Charles, 2010). Therefore, the development of morphological defensive traits involves a series of changes in the expression of chitin, hormone, and epidermal formation genes at different times (Christjani, Fink, & Elert, 2016; Miyakawa et al., 2010) as well as the regulation of epidermal cell growth via endocrine hormones (Weiss, Leese, Laforsch, & Tollrian, 2015). In the present study, significant changes in the expression of genes and pathways involved in cuticle protein, cutin, suberine, and wax biosynthesis as well as steroid hormone biosynthesis were noted. The expression of these genes may promote the synthesis of related substances (Fig. 5) and regulate individual growth (Edgar, 2006). However, the growth and development of cladocerans requires continuous molting and formation of a new carapace; thus, the maintenance of horns requires continuous substance synthesis, which may result in constant distribution costs (Auld et al., 2010). Furthermore, in *C. cornuta* , *Chaoborus* predation risk altered digestion and absorption by modulating the expression of the trypsin gene, which may affect the digestion and resource allocation strategies (Von Elert et al., 2004).

Smaller size, earlier reproduction, and increased brood number are adaptive responses to fish predation risks, which are similar to the typical responses of other cladocerans under fish predation (Diel et al., 2020). In terms of gene expression, our results showed that genes encoding actin and ribosomal proteins were down-regulated under fish predation risks. Since actin plays an important role in cytoskeletal structure, its inhibition may result in a smaller size. Similar results have been reported in previous studies on inducible defense responses of*D. magna* (Effertz et al., 2015; Pijanowska & Kloc, 2004). On the contrary, Schwarzenberger, Courts, and Von Elert (2009) revealed that the actin genes in *D. magna* were up-regulated under fish predation risks. Since gene expression is jointly regulated by transcriptional regulators and related proteins (Stibor, 2002), differential expression patterns could be observed at different time points (Effertz & Von Elert, 2014). Ribosomal proteins are responsible for protein assembly and translation; thus, the down-regulation of ribosomal protein may inhibit the synthesis of proteins essential for individual growth and development of *C. cornuta* (Zhou, Liao, Liao, Liao, & Lu, 2015). In enrichment analysis, some DEGs were found to be enriched in multiple pathways. Significantly enriched phototransduction altered the visual perception of *Daphnia*(Mahato et al., 2014), which may be an adaptation to behavioral responses, such as habitat selection (Loose & Dawidowicz, 1994) and escape behavior (Pietrzak, Pijanowska, & Dawidowicz, 2017). In*Daphnia* , fish predation reduced unsaturated fatty acid levels in neonates, rendering them vulnerable to starvation (Stibor & Navarra, 2000); therefore, the significantly enriched unsaturated fatty acid pathways may alter the distribution of these components. Furthermore, the longevity regulating pathway was significantly enriched under fish predation risks, which may incur an opportunity cost, that is, a decline in lifespan (Dawidowicz, Predki, & Pietrzak, 2010).

Under different predation risks, *C. cornuta* showed general responses, such as the expression of cysteine protease, heat shock protein, actin, and tubulin genes. The cDNA sequence of crustacean cysteine is similar to that of insect cathepsin L, which regulates the molting cycle and promotes cell death during development (Agrawal, Bagchi, & Bagchi, 2005). Thus, cysteine protease likely affected molting and increased brood number in the present study. The up-regulation of heat shock proteins is an adaptive response to various environmental stresses, including predation risks (Pijanowska & Kloc, 2004). As this response is rapid and returns to the previous state after long-term treatment (Pauwels, Stoks, & De Meester, 2005; Pauwels, Stoks, Decaestecker, & De Meester, 2007), the down-regulation of heat shock protein genes likely promoted the recovery of heat shock proteins in the present study. Similarly, general responses of the actin and tubulin genes, which are involved in cytoskeleton formation and other life activities have been observed in *Daphnia* (Pijanowska & Kloc, 2004), although their specific functions warrant further research (Chen et al., 2018).

Based on the classification of inducible responses, we further analyzed the early transcriptional data of *Daphnia magna* in response to vertebrate (sticklebacks) and invertebrate (*Triops*) predation risks (Orsini et al., 2016). The results are consistent with our finding that unique responses to different predators are dominant, while antagonistic responses are rare (Fig. 6 and Table S6). Moreover, previous weighted gene co-expression network analysis revealed that the most significantly co-expressed gene networks under vertebrate and invertebrate predation risks were unique (Orsini et al., 2018). Thus, diverse predator-dependent unique responses are favored by cladocerans during their co-evolution with multiple predators. For successful evolution of diverse predator-dependent unique responses, genotype, selection, and cost are important. First, the genotypes of cladocerans in ponds or lakes are highly diverse and the inducible traits of different clones are uncoupled (Boersma, Spaak, & De Meester, 1998; Decaestecker, De Meester, & Mergeay, 2009; Stoks, Govaert, Pauwels, Jansen, & De Meester, 2016). Second, multiple predators produce variable selection effects, which contribute to predator-dependent unique defensive traits (Herzog & Laforsch, 2013; Heynen, Bunnefeld, & Borcherding, 2017). Finally, environmental costs, such as altered predator regimes, may exceed maintenance costs (Decaestecker et al., 2002; Tollrian, 1995; Yin, Laforsch, Lohr, & Wolinska, 2011). Therefore, diverse predator-dependent unique responses are favored by prey.

In the present study, prey exhibited coupled responses to the same predator but extremely weakly coupled predator-dependent unique responses to antagonistic predation risks. Evidently, prey can alter resource allocation strategies under a single type of predation risk, resulting in an array of adaptive responses (Reede, 1995). In addition, the co-expression of uncoupled unique inducible responses can help prey avoid the dilemma of which predator to respond to, thus improving their survival rate under antagonistic predation risks. For instance, smaller *C. cornuta* individuals are less likely to be found by fish (O'Brien, 1987). Simultaneously, horn expression renders *C. cornuta* less vulnerable to predation by *Chaoborus* larvae (Gu et al., 2021). However, the effects of joint predation risks on the expression of predator-dependent unique responses are prominent and could be influenced by development, indicating a complex trade-off underlying adaptation to multiple predation risks (Riessen & Gilbert, 2019). Therefore, further studies are warranted to elucidate the mechanisms of the phenomenon that diverse inducible responses of prey can be elicited simultaneously under joint predation risks.

Conclusions

Through responses of individual traits and transcriptome, the present study revealed the inducible responses of *C. cornuta* to predation by *Chaoborus* larvae and fish. To cope with such antagonistic predation risks, *C. cornuta* mainly altered cuticle gene expression and developed horns under *Chaoborus* predation risk, while it altered ribosome gene expression and reduced body size under fish predation risk. Our analysis of these inducible responses revealed for the first time that predator-dependent unique responses are dominant and antagonistic responses are rare, implying that predator-dependent unique responses are favored by cladocerans during their long-term co-evolution with multiple predators, thereby lowering the environmental cost of inducible defenses. However, unique responses to one predator cannot completely avoid their dilemma of responding to other predators, although this dilemma is relatively limited. The present study expands

our understanding of the evolution and expression of inducible defenses under multiple predation risks.

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Data Accessibility

The raw data was uploaded to the NCBI Sequence Read Archive (SRA) and the BioProject ID is PRJNA735795.

Author contributions

LG, SA, LZ, and ZY designed the experiment. LG, SQ, YS, and JH performed the experiment and analyzed the data. LG and ZY wrote the first draft of the manuscript. All authors participated in discussions and editing of the manuscript.

Tables and Figures

Tables

Table 1. Results of MANOVA, two-way ANOVA, and Scheirer–Ray–Hare test on different traits of *C. cornuta* . Significant results ($P < 0.05$) are indicated in bold.

MANOVA	MANOVA	*Chaoborus* larvae (CH)	*Chaoborus* larvae (CH)	*Chaoborus* larvae (C
		F	*df*	Pillai
All traits	All traits	26.615	9	0.952
Two-way ANOVA	Two-way ANOVA	*F*	*df*	*P*
Size at maturity	Size at maturity	3.811	1, 35	0.059
Size at 16th day	Size at 16th day	4586	1, 34	**<0.001**
Neonate size	Neonate size	0.368	1, 20	0.551
Time to first brood	Time to first brood	1.041	1, 35	0.315
Total offspring number	Total offspring number	15.158	1, 34	**<0.001**
Brood number	Brood number	2.269	1, 34	0.141

MANOVA	MANOVA	*Chaoborus* larvae (CH)	*Chaoborus* larvae (CH)	*Chaoborus* larvae (C
Average brood size	Average brood size	10.626	1, 34	**0.003**
Scheirer-Ray-Hare	Scheirer-Ray-Hare	*H*	*df*	*P*
Horns at maturity	Horns at maturity	24.743	1, 34	**<0.001**
Horns at 16th day	Horns at 16th day	12.46	1, 34	**<0.001**

Table 2. Key genes involved in significant functional pathways in response to fish and *Chaoborus* predation risks.

	Function pathway	*P* value	*P* value	ID	Gene name	Log_2 (Change ratio) by transcriptomics	Log_2 (Change ratio) by transcriptomic
		C vs. CH	C vs. F			CH/C	F/C
Unique responses to *Chaoborus* predation risk	Steroid hormone biosynthesis	<0.001	-	Unigene0036707	UDP-glucuronosyltransferase 2A1	1.358	-0.025
				Unigene0033497	Estradiol 17-beta-dehydrogenase 12	1.330	-0.097
	Protein digestion and absorption	0.001	-	Unigene0036164	Trypsin	-1.379	-0.744
				Unigene0029770	Monocarboxylate transporter	-2.637	-0.207
				Unigene0033452	Proton-coupled amino acid transporter	1.816	-0.333
	Cutin, suberine and wax biosynthesis	0.018	-	Unigene0030892	Fatty acyl-CoA reductase 1	1.776	0.270
	Others	-	-	Unigene0000377	Cuticle protein	-2.935	-0.277
Unique responses to fish predation risk	Cysteine and methionine metabolism	-	<0.001	Unigene0005204	adenosylhomocysteinase	-0.993	-1.084
				Unigene0027903	aspartokinase	-1.041	-1.196
	Ribosome	-	0.001	Unigene0031920	60S ribosomal protein L18a	-0.501	-2.000

	Function pathway	P value	P value	ID	Gene name	Log_2 (Change ratio) by transcriptomics	Log_2 (Change ratio) by transcriptomic
				Unigene0015781	60S ribosomal protein L23a	0.241	-3.459
				Unigene0033039	40S ribosomal protein S3a	-0.481	-1.445
				Unigene0021976	ribosomal protein L13	-0.716	-1.414
	Phototransduction	-	0.004	Unigene0000385	actin	-0.626	-1.363
				Unigene0009756	Calmodulin	-0.400	-3.985
	Biosynthesis of unsaturated fatty acids	-	0.022	Unigene0026611	short-chain type dehydrogenase	-0.776	-4.644
	Longevity regulating pathway	-	0.028	Unigene0032764	Glutathione S-transferase	-0.501	-5.066
General responses	Apoptosis	0.002	0.003	Unigene0019346	cysteine proteinase	-1.389	-1.506
				Unigene0016049	HSP70	-1.302	-1.404
	Antigen processing and presentation	0.008	0.007	Unigene0000044	actin	-1.522	-1.237
				Unigene0031360	alpha-tubulin	-1.107	-1.477

Figures

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Figure 1 . Morphology and life history responses of *C. cornuta* under fish and *Chaoborus* predation risks. The dark line with asterisks indicates significant differences between treatments with and without fish predation risk (*$P < 0.05$, **$P< 0.01$, and ***$P < 0.001$). The upper- and lowercase letters indicate differences among treatments without and with *Chaoborus* predation risk, respectively. The error bar indicates standard error.

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ceriodaphnia-cornuta

Figure 2 . Correlation analysis (A) and principal component analysis (B) of gene expression in *C. cornuta* under different predation risks. C: control; CH: *Chaoborus* predation risk; F: fish predation risk.

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Figure 3 . Quantitative analysis of differentially expressed genes of *C. cornuta* under different treatments. Abbreviations of different treatments are the same as in Figure 2.

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Figure 4 . Correlations of different responses of individual traits (A) and transcriptional expression (B). Asterisks indicate significant Pearson's correlations between two traits or genes ($P< 0.05$). The correlations between unique responses to different predators are in squares. HM: horns at maturity; H16D: horns at 16th day; TON: total offspring number; TFB: time to first brood; NS: neonate size; SM: size at maturity; BN: brood number; S16D: size at 16th day; ABS: average brood size.

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Figure 5 . Diagram of *C. cornuta* pathways in response to fish and *Chaoborus* predation risks.

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Figure 6. Quantitative analysis of differentially expressed genes of *Daphnia magna* under fish (sticklebacks) and *Triops* predation risks. The data are from the work of Orsini et al. (2016). Detailed results are presented in Table S6. Clones I and X were collected from a system of ephemeral rock pools in Southwest Finland and a fish-rearing pond in Southern Germany, respectively. CO: control; FI: fish predation risk; TR: *Triops* predation risk.

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