Inducible responses to antagonistic predation risks are not in a dilemma: Evidences from multi-traits and transcriptome of Ceriodaphnia

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Abstract

Inducible defenses of prey are evolved under diverse and variable predation risks. In the co-evolution of prey and multiple predators with antagonistic selection pressures, whether inducible defense responses of prey will fall into a dilemma and its underlying mechanism are still unclear. Based on the antagonistic predation pressure from invertebrate predator Chaoborus larvae and vertebrate predator fish, we studied multi-traits and transcriptome of the freshwater crustacean Ceriodaphnia cornuta under multiple predation risks. Our results showed that Chaoborus larvae predation risks altered the expression of genes encoding cuticle protein and changed the biosynthesis of steroid hormone, cutin, suberine, and wax, promoting Ceriodaphnia to express horns and grow larger at a late development stage, whereas fish predation risks mainly triggered responses in genes encoding ribosome and pathways of unsaturated fatty acids biosynthesis, cysteine and methionine metabolism, resulting in a smaller individual size and earlier reproduction. The inducible responses on transcription and individual traits both revealed that predator unique responses are dominant and the antagonistic responses are the least. Besides, Pearson correlations between different predator unique responses are extremely weak. Furthermore, the unique individual traits triggered by different predators can be expressed simultaneously. These results indicated that Ceriodaphnia can avoid the dilemma by performing predator unique responses and diverse inducible responses are favored in the co-evolution of zooplankton and multiple predators.

Inducible responses to antagonistic predation risks are not in a dilemma: Evidences from multi-traits and transcriptome of *Ceriodaphnia*

Running title: Inducible responses to multiple predators

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Abstract

Inducible defenses of prey are evolved under diverse and variable predation risks. In the co-evolution of prey and multiple predators with antagonistic selection pressures, whether inducible defense responses of prey will fall into a dilemma and its underlying mechanism are still unclear. Based on the antagonistic predation pressure from invertebrate predator *Chaoborus* larvae and vertebrate predator fish, we studied multi-traits and transcriptome of the freshwater crustacean*Ceriodaphnia cornuta* under multiple predation risks. Our results showed that *Chaoborus* larvae predation risks altered the expression of genes encoding cuticle protein and changed the biosynthesis of steroid hormone, cutin, suberine, and wax, promoting*Ceriodaphnia* to express horns and grow larger at a late development stage, whereas fish predation risks mainly triggered responses in genes encoding ribosome and pathways of unsaturated fatty acids biosynthesis, cysteine and methionine metabolism, resulting in a smaller individual size and earlier reproduction. The inducible responses on transcription and individual traits both revealed that predator unique responses are dominant and the antagonistic responses are the least. Besides, Pearson correlations between different predator unique responses are extremely weak. Furthermore, the unique individual traits triggered by different predators can be expressed simultaneously. These results indicated that *Ceriodaphnia* can avoid the dilemma by performing predator unique responses and diverse inducible responses are favored in the co-evolution of zooplankton and multiple predators.

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

Introduction

In the co-evolution of predator and prey, defense is critical to the survival of prey. Because of the changing predation risks, inducible defenses, triggered by predation cues, are favored by prey (Tollrian & Harvell, 1999). To successfully defend predators, prey organisms can perform various inducible defensive traits, including behavior (De Meester, 1993), morphology (Gu et al., 2021), chemical substance (Selander et al., 2015), and life history (Kvile, Altin, Thommesen, & Titelman, 2021). Since diverse predation risks can prevent the stable expression of an inducible defensive trait (Steiner & Auld, 2012), the present study seeks to understand how a prey response to multiple predation risks, especially to predators with 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 integration of inducible defense researches, the present study classified the responses against multiple predators into two major categories. The first type is the general response, which evolves through diffusion co-evolution and is a reciprocal adaptation to similar predators, for example, mayfly preforms the same avoidance behavior under different fish predation risks (Alvarez, Landeira-Dabarca, & Peckarsky, 2014); the other type is the specific response, which is evolved by pairwise co-evolution between specific predator and prey, such as the immune responses of immune systems to pathogens (Westra et al., 2015) and the inducible crests of *Daphnia* in response to*Notonecta* predation (Grant & Bayly, 1981). By considering the specific responses in different traits, they can be further subdivided into antagonistic responses on the same trait and unique responses on separate traits. Assuming the selection pressures are antagonistic, if the prey mainly performs antagonistic responses on the same traits, then inducible responses to one predator may cause the prey suffer an environmental cost, i.e., vulnerable to the other predator; if the unique responses on separate traits dominate, then the complex defense responses may create maintenance costs, i.e., energetic costs of the sensory and regulatory mechanisms (Auld, Agrawal, & Relyea, 2010). Based on this, we cannot theoretically speculate whether inducible responses of prey against antagonistic predation risks are limited and in a dilemma.

In aquatic ecosystems, cladocerans are in the middle of the food chain, providing food resources 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 example, larger

plankton is vulnerable to the large visual predator fish, while less vulnerable to the small ambush predator *Chaoborus* larvae (Swift, 1992). Thereby, antagonistic inducible traits are commonly expressed by *Daphnia* , for example, *Daphnia hyalina* shows completely opposite response in size and reproduction under fish and invertebrates predation pressure (Stibor & Lüning, 1994); *Daphnia galeata*prefers deeper habitat under fish predation risk, while inhabits the upper layer under *Chaoborus* larvae predation risk (Dodson, 1988). Besides, unique inducible defensive traits are prominent in*Daphnia* , such as "twist" (Herzog, Rabus, Ribeiro, & Laforsch, 2016), neck teeth (Tollrian, 1993), and horns (Gu, Qin, Zhu, et al., 2020). Furthermore, general responses are noticeable, such as the elongated tail spine, which is observed in *Daphnia* in response to fish, *Triops* , and *Notonecta* (Gu, Qin, Lu, et al., 2020; Ritschar, Rabus, & Laforsch, 2020). Consistent with the summary of inducible defensive traits, both general and specific responses appear at molecular levels, for instance, *Daphnia magna* decreases actin and tubulin expression under the predation risk of *Chaoborus*larvae or fish (Pijanowska & Kloc, 2004), increases the expression of ribosomal protein and vitellogenin under fish predation risks (Effertz, Mueller, & von Elert, 2015), while decreases the expression of vitellogenin and increases cuticle protein under *Triops* predation risks (Otte, Fröhlich, Arnold, & Laforsch, 2014). Therefore, in the summary of *Daphnia* researches, this typical research organism shows various types of responses under antagonistic predation risks. However, through these scattered studies on different *Daphnia*species and clones, we still cannot conclude which type of response is preferred by a prey.

Ceriodaphnia cornuta is a widely distributed species with sensitive inducible defensive traits (Gu et al., 2021; Qin et al., 2021), providing a suitable organism for answering the above question. Since some inducible traits are hidden (Laforsch, Ngwa, Grill, & Tollrian, 2004), researches on a few traits are not sufficient. In recent years, omics technologies promote our understanding of the mechanisms of inducible defenses (Hales et al., 2017; Zhang et al., 2021). Therefore, to systematically answer how a prey response to antagonistic predation risks, the present study tested multi-traits and transcriptome of *C. cornuta* in response to *Chaoborus*larvae and fish. Besides, to better understand the strategy of inducible responses, we analyzed the Pearson correlation between different inducible responses.

Materials and methods

Predation risks

The predation risks were simulated by different predator-conditioned medium, which was prepared according to Gu, Qin, Zhu, et al. (2020). We cultured 4 *Rhodeus ocellatus* or 100 *Chaoborus* sp. larvae in aged tap water and fed enough *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 stock predator-conditioned medium, containing different predator kairomones (Hahn, Effertz, Bigler, & von Elert, 2019; Weiss et al., 2018), were filtered through a 0.22 μm glass fiber filter (Millipore) and then the filtrates were stored in a refrigerator before the experiments. To test the response of*Ceriodaphnia* against fish, as well as *Chaoborus* , we set up a full factor experiment containing the following treatments: The control (C) treatment was COMBO medium; Fish (F) and *Chaoborus*(CH) predation risk treatments were produced by diluting their filtered stock medium 20 times (i.e., 1 fish per 10 L) and 2.5 times (i.e., 20*Chaoborus* larvae per L) in COMBO medium, respectively; the combination treatment (CH + F) consisted of the above two diluted medium.

Life history experiment

The *C. cornuta* clone used in the present study was sampled from Lake Taihu (31°22'13.548"N, 120deg0'16"E), China. We cultured *C. cornuta* in COMBO medium and fed with *Chlorella pyrenoidosa* (1.5 mg C/L) at 25 degC under a fluorescent light intensity 500 Lux in a 14:10 h light/dark cycle. Synchronous *C. cornuta* with a density of 1 ind per 10 mL were adapted to the above conditions for at least two generations. We randomly divided newborn individuals into different treatments within 12 h. Each individual was cultured in 10 mL medium with 10 replicates for each treatment and the media in different treatments were refreshed daily.

The body size and horns were detected at maturity and a late developmental stage, i.e., the 16th day. We scored the horns of *C. cornuta* according to Gu et al. (2021), i.e., absent (score 0), small (score 5), and large (score 10), and then the individual scores were normalized by a maximum point to define the induction levels between 0% and 100%. Besides, time to the first brood, neonate size, brood number, total offspring number, and average brood size were recorded in the present study.

RNA samples and sequencing

To further analyze the type of responses on the transcriptional level, we sequenced the transcriptome of *C. cornuta* under C, F, and CH treatments. Groups of 250 newborn individuals were cultured in 2.5 L medium with 3 replicates for each treatment. During this cultivation, responses triggered by different predation risks were verified through inducible traits, i.e., horns and body size at maturity. We refreshed the medium daily and took samples within 12 h after the first brood of*C. cornuta* . *Ceriodaphnia* samples were frozen in liquid nitrogen and homogenized in TransZol Up, and the total RNA was extracted using TransZol Up Plus RNA Kit following the manufacturer's instructions (ER501, TRANS, China). RNA quality was assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and checked by agarose gel electrophoresis. In the present study, the RNA integrity number of all samples was above 7.0.

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

Transcript assembly and annotation

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

Quantification of gene expression and RT-qPCR Analysis

The unigene expression was calculated and normalized to RPKM (Reads Per kb per Million reads) (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008). RNAs differential expression analysis was performed by DESeq2 software between control and different predation risk treatments. The genes with the parameter of false discovery rate (FDR) below 0.05 and absolute fold change [?] 1 were considered differentially expressed genes (DEGs). We classified the DEGs into different types according to our classification and then used KEGG analysis to classify the function of DEGs. Pathways with P -value [?] 0.05 were considered as significantly enriched pathways. The representative DEGs of different significantly enriched pathways were selected according to the following precedence conditions: stable expression, large fold change, and the pathways are annotated on a closely related species, i.e., *D. magna* and *D. pulex* .

To validate the RNA-Seq data in *C. cornuta* transcriptome, we quantitated the expressions of 18 random DEGs by RT-qPCR. ddH2O was used as the negative control in RT-qPCR. The cDNA was synthesized from mRNA by Reverse Transcriptase SuperMix (R233, Vazyme, China), and RT-qPCR was conducted using ChamQ Universal SYBR qPCR Master Mix (Q711, Vazyme, China). All the primer sequences are presented in Supplementary Table 1. We obtained expression data from four alternative reference genes (Scoville &

Pfrender, 2010) and calculated their average gene expression stability by geNorm. The glyceraldehyde-3-phosphate dehydrogenase (G3PD), the RNA polymerase II gene (RNAP II), and the elongation factor 1-alpha gene (EF) were determined to be stably expressed and were geometrically averaged to calculate a gene expression normalization factor for each sample. Gene expression was calculated using $2^{-\Delta\Delta\tau}$ method. Correlation between RNA-Seq and RT-qPCR was performed by regression analysis.

Statistical Analysis

To test the effects of different predation risks, a MANOVA, followed by two-way ANOVA, was performed on individual traits. The significant differences ($P < 0.05$) among different treatments were tested by the Bonferroni test. When the normality test (Shapiro-Wilk) failed, we used Scheirer-Ray-Hare followed by Wilcoxon rank-sum test to analyze the differences between treatments. To test the relationship between different types of response, we analyzed the Pearson correlation coefficient between traits, as well as representative DEGs, in different categories. The statistical tests were performed using R software (version 3.6.2).

Results

Morphology and life history traits

Different predation risks significantly triggered various responses in morphology and life history traits (Table 1). Compared with control, the responses induced by fish and *Chaoborus* larvae 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, i.e., *C. cornuta* expressed horns (maturity: $P < 0.001$; 16th day: $P < 0.001$) and increased offspring number ($P = 0.002$) under *Chaoborus* larvae predation risk, and the traits were not significantly changed by fish predation risk. (2) Unique responses to fish: time to first brood and neonate size, i.e., the neonate size ($P = 0.006$) and time to first brood ($P< 0.001$) of *C. cornuta* were remarkably decreased under fish predation risk, and these responses were not significant under *Chaoborus* larvae predation risk. (3) General responses: Size at maturity and brood number, i.e., 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 fish and *Chaoborus* larvae predation risks. (4) Antagonistic responses: size at 16th day, i.e., *C. cornuta* increased the size under *Chaoborus* larvae ($P = 0.034$), but decreased the size under fish predation risks ($P < 0.001$). Additionally, no significant differences were observed in average brood size. Among all those traits, unique responses to predators were dominant, i.e., unique responses (5) > general responses (2) > antagonistic responses (1).

Interactions between fish and *Chaoborus* larvae predation risks were noticeable in different traits (Table 1). General responses: size at maturity in the combination treatment was smaller than that in control ($P <0.001$) and had no significant difference with that in fish predation risk treatments; brood number of the combination treatment did not have remarkable differences with that of other treatments. Unique responses: time to first brood (CH + F vs. C:$P = 0.027$) and neonate size (CH + F vs. C: $P = 0.003$) were significantly decreased in the combination treatment and had no significant differences with those in fish predation risk treatment; horn expression in the combination treatment was significant at maturity (CH + F vs. C: $P = 0.007$), while this inducible trait was greatly impaired when compared with that of *Chaoborus* larvae predation risk treatment (maturity: $P = 0.012$; 16th day: $P < 0.001$). Antagonistic responses: size at 16th day in the combination treatment was significantly smaller than that in *Chaoborus* larvae predation risk treatment ($P < 0.001$), higher than that in fish predation risk treatment ($P = 0.065$), and not different from that in control ($P = 0.764$).

Overview of assembled transcriptome

We obtained 78341484, 72493072, and 69930191 clean reads in C, CH, and F, respectively. A total of 37120 assembled unigenes were assembled through Trinity software and each sample contained more than 66.54% assembled unigenes (Supplementary Table 2). By comparing with Nr, KEGG, COG, and SwissProt

databases, a total of 18343 unigenes (49.4%) were annotated in these public databases (Supplementary Fig. 1). Regarding the species distribution in the Nr database, *C. cornuta* had the highest comparison rate with *Daphnia magna* (26.33%), followed by *D. pulex* (2.6%) (Supplementary Fig. 2A); the unigenes enriched in the KOG database were classified into transcription, ribosomal structure, and gene replication, recombination, and repair (Supplementary Fig. 2B); the annotated GO terms were mainly associated with metabolic process, cellular process, cell part, and binding (Supplementary Fig. 2C).

Differentially expressed genes (DEGs)

Paired samples within the same treatment had high Pearson correlation coefficients ([?]0.90) and were clustered together in principal component analysis (Fig. 2A and B), which conformed to requirements of biological repetition. Furthermore, the expression patterns showed a significant correlation between RT-qPCR and RNA-Seq (Fig. 2C (a)$R^2 = 0.936$; (b) $R^2 = 0.788$), indicating that our analysis on the expression data by RNA-Seq is reliable.

Compared with control, *Chaoborus* larvae and fish predation risk significantly affected the expression of 1515 and 846 genes, respectively (Fig. 3A). Among them, there were 1399 unique DEGs in CH, 730 unique DEGs in F, 114 general DEGs, and 2 antagonistic DEGs (Fig. 3B). Therefore, unique responses to predators were dominant at the transcriptional level, i.e., unique DEGs (2129) > general DEGs (114) > antagonistic DEGs (2) (Supplementary Table 3).

Considering the DEGs caused by different predators, we further analyzed the differences in the enriched pathways of *C. cornuta* (Supplementary Table 4) and concluded the main DEGs and pathways related to inducible defensive traits (Table 2). The DEGs of *C. cornuta* against *Chaoborus* larvae, 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; the DEGs of *C. cornuta* against fish, containing ribosomal protein, actin, and short-chain type dehydrogenase genes, were mainly enriched in cysteine and methionine metabolism, ribosome, phototransduction, and biosynthesis of unsaturated fatty acids; the general DEGS, including cysteine proteinase, HSP70, actin, and alpha-tubulin genes, were enriched in the pathways of apoptosis and antigen processing and presentation.

Correlation analysis between different inducible responses

Both 9 individual traits and 40 representative DEGs (Supplementary Table 5) revealed high Pearson correlations within unique responses to fish or *Chaoborus* larvae (Fig. 4), such as the response of horns at maturity was significantly correlated with the changes of horns at 16th day ($P = 0.05$) and total offspring number ($P = 0.05$). Besides, both correlation analyses showed weak Pearson correlation coefficients between unique responses to fish and unique responses to *Chaoborus* larvae. While, a few significant correlations appeared on the weak correlation area in the transcriptional expression, such as Unigene0008297, in ribonucleoprotein component protein expression, had significant positive relationships with Unigene0027903 ($P= 0.049$) and Unigene0005204 ($P= 0.013$) in Aspartokinase and Adenosylhomocysteinase expression, respectively (Fig. 4; Supplementary Table 5).

Discussion

Our experiments clearly showed that *C. cornuta* performed diverse responses under the antagonistic predation risks consisted of fish and *Chaoborus* larvae. Based on the classification of inducible responses, our results revealed, for the first time, that predator unique responses are dominant, followed by the general responses, and the antagonistic responses are the least. When the antagonistic predation risks coexist, unique individual traits triggered by different predators can be expressed simultaneously, thereby the Pearson correlations between the unique responses to different predators are very weak. According to these results, this study supports the view that prey prefers predator unique responses in the co-evolution of prey and multiple predators, which may cause complex costs and limitations of inducible defenses.

Horns and larger size are adaptive inducible traits to *Chaoborus*larvae predation (Gu et al., 2021; Riessen & Trevett-Smith, 2009). The larger individual size at a late development stage requires rapid growth and more food intake (Gianuca, Pantel, & De Meester, 2016), thereby promoting the brood number and total offspring number. The horns are formed by the carapace, which is composed of two layers of dermal cells and covered by chitin, which combines with cuticle protein (Charles, 2010). Therefore, the formation of morphological defensive traits involves a series of changes in the expression of chitin, hormones, and epidermal formation genes at different times (Christjani, Fink, & Elert, 2016; Miyakawa et al., 2010), as well as the regulation of epidermal cell growth by endocrine hormones (Weiss, Leese, Laforsch, & Tollrian, 2015). In this study, significant changes in genes and pathways were involved in cuticle protein, cutin, suberine, and wax biosynthesis, and steroid hormone biosynthesis. These genes' expression may promote the synthesis of related substances (Fig. 5) and regulate individual growth (Edgar, 2006). However, growth and development of cladocerans require continuous molting and formation of new carapace, thus, the maintenance of horns needs continuous substance synthesis, which may result in continuous distribution costs (Auld et al., 2010). Besides, *Chaoborus* predation risks altered the digestion and absorption of *C. cornuta* , such as the trypsin gene, which may affect the digestion and resource allocation strategy (Von Elert et al., 2004).

The 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 expressions, our results showed that the genes encoding the proteins of actin and ribosomal are down-regulated under fish predation risks. Since actin plays an important role in the structure of the cytoskeleton, the inhibition of actin may result in a smaller cladoceran size. Similar results were observed in the studies of*D. magna* inducible defenses (Effertz et al., 2015; Pijanowska & Kloc, 2004). On the contrary, Schwarzenberger, Courts, and von Elert (2009) revealed an up-regulation of actin genes in *D. magna* under fish predation risks. Because gene expression is jointly regulated by transcriptional regulators and related proteins (Stibor, 2002), the differential expressions could be observed within 1-2 hours (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 that are needed for individual growth and development (Zhou, Liao, Liao, Liao, & Lu, 2015), ultimately affecting the growth of *C. cornuta* . In the enrichment analysis, some DEGs can be enriched in multiple pathways. The significantly enriched phototransduction may change the visual perception of*Daphnia* (Mahato et al., 2014), which could be an adaption to behavioral responses, such as habitat selection (Loose & Dawidowicz, 1994) and escape behavior (Pietrzak, Pijanowska, & Dawidowicz, 2017). Besides, fish predation can reduce the unsaturated fatty acids of neonates, causing *Daphnia* to be vulnerable to starvation (Stibor & Navarra, 2000), therefore the significantly enriched pathway of unsaturated fatty acids may alter the distribution of unsaturated fatty acids. Furthermore, the longevity regulating pathway was significantly enriched under fish predation risks, which may cause an opportunity cost, i.e., the decline of lifespan (Dawidowicz, Predki, & Pietrzak, 2010).

When facing different predators, *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 protein is closer to that of insect cathepsin L, which regulates the molting cycle and programs cell death during development (Agrawal, Bagchi, & Bagchi, 2005). Thus, the affected cysteine protease may affect molting and increase brood number in the present study. The up-regulation of heat shock protein is an adaptive response various environmental stresses, including predation risks (Pijanowska & Kloc, 2004). Because this response is rapid and returns to previous level after long-term treatment (Pauwels, Stoks, & De Meester, 2005; Pauwels, Stoks, Decaestecker, & De Meester, 2007), the down-regulation of heat shock protein genes may promote the recovery of heat shock protein in this study. Similarly, general responses are observed in actin and tubulin genes of *Daphnia* (Pijanowska & Kloc, 2004), they are involved in the formation of the cytoskeleton and other life activities, while their specific functions still need further researches (Chen et al., 2018).

From the perspective of different responses, prey performs coupling responses to the same predator and extremely weak coupling unique responses to antagonistic predation risks. It is easy to understand that prey can alter resource allocation strategies under single predation risk, resulting in an array of adaptive responses

(Reede, 1995). For a successful evolution of predator unique responses under multiple predation risks, we mainly considered it from the genotype, selection, and cost. Firstly, 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). Secondly, in the process of predation, multiple predators have diversified selection effects, which contribute to predator unique defensive traits (Herzog & Laforsch, 2013; Heynen, Bunnefeld, & Borcherding, 2017). Finally, the environmental costs, such as changing predator regimes, may exceed maintenance costs (Decaestecker, De Meester, & Ebert, 2002; Tollrian, 1995; Yin, Laforsch, Lohr, & Wolinska, 2011), thus, complex unique responses are favored by prey. In our study, this inducible defensive strategy can avoid the dilemma of responses on single traits, improving the survival rate of prey under multiple predation risks. For example, smaller *C. cornuta* is less likely to be found by fish (O'brien, 1987). At the same time, horn expression makes *C. cornuta* less vulnerable to*Chaoborus* larvae predation (Gu et al., 2021). While, the co-expression of unique inducible defenses is influenced by development, indicating that there is a trade-off underlying the adaption to multiple predation risks (Riessen & Gilbert, 2019). Therefore, further studies are still needed to reveal how prey responses to multiple predators, especially in a complex biological and abiotic environment.

Conclusions

Through the responses on individual traits and transcription, this study revealed inducible responses of *C. cornuta* against*Chaoborus* larvae and fish. To cope with such antagonistic predation risks, *C. cornuta* mainly changed cuticle gene expression and formed horns under *Chaoborus* larvae predation risk, while altered ribosome genes expression and reduced body size under fish predation risks. Our analysis on those inducible responses revealed for the first time that different predator unique responses are dominant and extremely weak coupling. Contrary to the dilemma of responses on a few inducible defensive traits, this study supports the view that zooplankton prefer predator unique responses and performs the least antagonistic responses in the adaption to antagonistic predation pressures, implying that the potential co-evolutions with multiple predators are mutually shaping the inducible responses of zooplankton.

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Data Accessibility

Sequence data is uploaded to the Sequence Read Archive (SRA) with accession 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 the MANOVA, Two-way ANOVA, and Scheirer-Ray-Hare on different traits of *C. cornuta* . Significant results ($P< 0.05$) are given 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
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 of significant function pathways in response to fish and *Chaoborus* larvae 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* larvae predation risks	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

	Function pathway	*P* value	*P* value	ID	Gene name	Log_2 (Change ratio) by transcriptomics	Log_2 (Change ratio) by transcriptomic
	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 risks	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
				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* larvae predation risks. The dark line with asterisks indicates significant differences between treatments with and without fish predation risks ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). The upper and lower case letters indicate differences among treatments of absent and present *Chaoborus* larvae predation risks, respectively. The error bar indicates the standard error.

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Figure 2 . Correlation analysis (A) and principal component analysis (B) of gene expression of *C. cornuta* under different predation risks. (C) Correlation between RNA-Seq and RT-qPCR data.

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Figure 3 . Quantitative analysis of differentially expressed genes of *C. cornuta* in different treatments.

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Figure 4 . Correlation relationships within different responses in individual traits and transcriptional expressions. The asterisks indicate significant Pearson correlations between two traits or genes ($P < 0.05$). The correlations between unique responses to different predators are in squares.

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