Effects of Short-time Exposure to Atrazine on miRNA Expression Profiles in the Gonad of Common Carp (*Cyprinus carpio*)

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ABSTRACT: MicroRNAs (miRNAs) are endogenous small non-coding RNAs that negatively regulate gene expression by targeting specific mRNAs; they are involved in the modulation of important mRNA networks involved in toxicity. Atrazine is a known endocrine-disrupting chemical, whose molecular mechanisms are unknown. In this study, common carp (*Cyprinus carpio*) gonads at two key developmental stages were exposed to 0.428 ppb atrazine for 24 h *in vitro*. MiRNA expression profiles were analysed to identify miRNAs related to

gonad development and to reveal the atrazine mechanisms interfering with gonad differentiation. Atrazine exposure caused significant alteration of multiple miRNAs. Compared with the juvenile ovary, more miRNAs were down-regulated in juvenile testis, some of these down-regulated miRNAs target the steroid hormone biosynthesis pathway related-genes. Predicted target genes of differently-expressed miRNAs after exposure to atrazine were involved in many reproductive biology signalling pathways. We suggest that these target genes may have important roles in atrazine-induced reproductive toxicity by altering miRNAs expression. Our results also indicate that atrazine can up-regulate aromatase expression through miRNAs, which supports the hypothesis that atrazine has endocrine-disrupting activity by altering the expression of genes of the Hypothalamus-Pituitary-Gonad axis through its corresponding miRNAs. This study tells us the following conclusions: 1. Atrazine exposure results in significant alterations of miRNAs whose predicted target genes are associated with reproductive processes. 2. In the primordial gonad, atrazine promoted the expression of early gonad-determining genes by decreasing specific miRNAs. 3. In the juvenile gonad, atrazine promoted the biosynthesis of steroid

hormones.

Keywords: Atrazine; targeting analysis; microRNA; gonad development; *Cyprinus carpio*

INTRODUCTION

Sex determination in fish is significantly influenced by environmental factors, such as temperature, pH, exogenous hormones, and pollutants (Devlin et al., 2002). Pollutants, such as pesticides, are potential endocrine disruptors, which even at very low levels are sufficient to cause developmental and reproductive alterations in numerous species (Colborn et al., 1993; Corcoran et al., 2010).

With the development of agriculture, herbicides are increasingly used to reduce soil erosion, to avoid the manual removal of weeds, and to increase crop production rates (Gianessi & Sankula, 2003). However, the use of pesticides leads to serious harm to living organisms. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a pre-emergent herbicide used on a variety of agricultural crops including corn, sorghum grass, sugar cane, and wheat (Barr et

al., 2007; Eldridge et al., 2008; Solomon et al., 2008). Atrazine is probably the most widely used herbicide in the world frequently contaminating potable water supplies (U.S. Environmental Protection Agency 1994). Atrazine is a suspected endocrine-disrupting chemical that alters male reproductive tissues, when animals are exposed during development.

Various studies indicate atrazine adversely impacts the neuroendocrine and reproductive systems, and that it may be a potential carcinogen (Cooper et al., 2007; Cragin et al., 2011; Hayes et al., 2010; Freeman et al., 2011). Currently the epigenetic, genetic, and cellular mechanisms altered by atrazine exposure are under investigation (Kucka et al., 2012; Karmaus and Zacharewski, 2015; Pogrmic et al., 2009, Pogrmic-Majkic et al., 2010, 2014; Wirbisky et al., 2016a,b). Tevera-Mendoza et al. (2002) showed that atrazine exposure, for as little as 48 h at 21 ppb, resulted in severe gonad dysgenesis in African clawed frogs (*Xenopus laevis*). Moreover, atrazine induced hermaphroditism at concentrations of only 0.1 ppb (Hayes et al. 2002). In fish, atrazine can result in complete feminization of males, as illustrated by skewed sex ratios in zebrafish (*Danio rerio*), which have no distinguishable sex chromosomes (Suzawa et al., 2008).

In zebrafish, atrazine exposure during embryonic development alters MicroRNAs (miRNAs) associated with angiogenesis, cancer, and neurodevelopment (Sara et al., 2016). Numerous studies have shown that atrazine has adverse effects on the neuroendocrine system, primarily affecting the hypothalamus–pituitary–gonad (HPG) axis. Atrazine decreases gonadotropin-releasing hormone release, the pre-ovulatory surge of luteinizing hormone, follicle stimulating hormone, and prolactin (Cooper et al., 2000; Foradori et al., 2009, 2013; Weber et al., 2013; Wirbisky et al., 2016a). However, the mechanism of action of atrazine is not well-understood.

MiRNAs are single-stranded, highly conserved, non-coding RNA molecules of 19–24 nucleotides (nt), which regulate gene expression at the post-transcriptional level, by targeting specific sites in the 3′ untranslated region of mRNAs (Bartel, 2004; He et al., 2004; Krol et al., 2010). miRNAs play important roles in controlling multiple biological processes, such as embryonic development, cell cycle control, apoptosis, cell proliferation and differentiation, and immune and stress responses in various organs (Brennecke et al., 2003; Hwang et al., 2006; Pedersen et al., 2007; Ro et al., 2007; Xu et al., 2003). In the last few years, miRNAs have been reported to play an important role in the

response to toxicant exposure and in the process of toxicant-induced tumorigenesis (Jardim et al., 2009; Rager et al., 2011; Zhang and Pan, 2009).

As a new tool for risk assessment, miRNAs can provide indications on the toxicology mechanisms associated with environmental factors and with disease. MiRNAs are also novel biomarkers of the diseases related to environmental factors (Li et al., 2014). Recently, an increasing number of studies have shown that miRNAs can functionally interact with a variety of environmental factors including drugs, viruses, radiation, and environmental chemicals (e.g., formaldehyde, PAHs, and bisphenol A) (Izzotti and Pulliero, 2014; Qiu et al., 2012; Ray et al., 2014). Knowledge on the role miRNAs in toxicological responses is increasing, but is still limited.

The common carp, *Cyprinus carpio*, is one of the most important cyprinid species, accounting for 10% of the global freshwater aquaculture production (Xu et al., 2014). Genomic studies of common carp have recently made extensive progress. Common carp transcriptome was deep sequenced by Ji et al. (2012) and Jiang et al. (2016), who identified changes at the transcriptomic level in common carp spleen after 24 h of experimental infection with *Aeromonas hydrophila*. A large

number of gene associated single-nucleotide polymorphisms (SNPs) were identified in four strains of common carp using nextgeneration sequencing (Xu et al., 2014). miRNAs and miRNA-related SNPs were also identified. MiRNA-related SNPs affect biogenesis and regulation in the common carp (Zhu et al., 2012).

Yellow River carp (common carp from the Yellow River) is famous in China for its tender, tasty, and nutritional meat. Females grow faster than males, which makes the mechanism of sex differentiation and development an intriguing topic in this commercially important species (Gui et al., 2012; Mei and Gui, 2015). In our previous study, we profiled miRNAs from five different developmental stages of Yellow River carp, in order to identify differentially-expressed and novel miRNAs that may play regulatory roles in ovary differentiation (Wang et al., 2017). Our previous study showed that there is a dynamic shift in gene expression during gonad differentiation and development. (Jia et al., 2017). Environmental factors can affect miRNAs in fish, and even play a decisive role in some species.

Several studies have shown that in zebrafish and humans atrazine exposure alters miRNAs associated with angiogenesis, cancer, and neurological development (Wirbisky et al., 2016).

However, few studies have investigated the role of miRNAs in toxicological responses during sex differentiation and development in teleost fish.

In this study, we looked for correlations of miRNA and mRNA expressions during sex differentiation and development of carp, following atrazine exposure. The gonad development of carp has several critical periods, including primordial gonad and juvenile gonad. It would be valuable to understand the gene expression changes and the roles of miRNAs during the key stages of gonad development of carp, when they are exposed to atrazine. Therefore we aimed to investigate the effect of atrazine exposure on the global expression profile of miRNAs in the two key stages of gonad development by deep sequencing. We also predicted target genes that would affect gonad development. Our results would help us to better understand the molecular mechanisms of atrazine toxicity on gonad development, and to reveal the roles of miRNA–mRNA interactions in toxicological mechanisms, and the important impact on sex differentiation and gonad development of common carp.

MATERIALS AND METHODS

Chemicals

Atrazine (purity > 98%) was purchased from Beijing Dezhong-Venture Pharmaceutical Technology Development Co., Ltd. (Beijing, China). As atrazine has low solubility in water, the stock solutions and dilutions were prepared in acetone (Fisher Scientific, USA) and stored at 4 °C.

Fish Samples

All investigations in this study were performed according to the Animal Experimental Guidelines of the Ethical Committee of the University of China. The Yellow River carp used in this study were obtained from the aquaculture facilities of Henan Normal University and maintained at the genetics laboratory (Henan normal university, Xinxiang Henan province, China) in flow-through water tanks with a constant temperature of 25 ± 1 ℃. The test samples included gonads from two different developmental stages. Samples of primordial gonads were collected from larvae at 45 days post-hatching, based on the results of our previous studies (Wang et.al, 2017). The original reproductive gland was dissected under a microscope, and samples from 50 fish were mixed after confirmation by histological section. Samples of juvenile gonad were collected from 30 fish 80 days post-hatching. Stage II ovaries and testis

were confirmed with histological sections.

Atrazine Exposure

Samples of two different stages including primordial gonad and juvenile gonad (ovary and testis) were cultured at 28 °C in a humidified 10% CO_2 atmosphere in Dulbecco's modified eagle medium supplemented with 10% foetal bovine serum (Gibco, Life Technologies) (Pombinho et al., 2004; Daniel et al., 2014). Culture medium was renewed every two days. For atrazine exposure experiments, cells were seeded in 24-well plates and allowed to proliferate for 48 h. Then samples were treated with 0.428 ppb of atrazine for 24 h. Three replicates were set for each treatment, as well as for the unexposed control. Samples were collected at 8 h and 24 h post-treatment, and were immediately frozen in liquid nitrogen and stored at −80 °C for further use.

RNA Isolation

Total RNA was extracted from each sample separately using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The quantity and purity of total RNA were checked using the Agilent 2100 Bioanalyzer system (Santa Clara, CA, USA) and by denaturing gel electrophoresis. The

221 samples were then stored at −80 °C.

222

223 **Small-RNA Library Construction and Sequencing**

224 We generated small-RNA libraries from the nine samples from
225 Yellow River carp: primordial gonad control (PG-CK),
226 primordial gonad exposed to atrazine for 8 h (PG-A8h),
227 primordial gonad exposed to atrazine for 24 h (PG-A24h),
228 juvenile ovary control (IIC-CK), juvenile ovary exposed to
229 atrazine for 8 h (IIC-A8h), juvenile ovary exposed to atrazine
230 for 24 h (IIC-A24h), juvenile testis control (IIX-CK), juvenile
231 testis exposed to atrazine for 8 h (IIX-A8h), juvenile testis
232 exposed to atrazine for 24 h (IIX-A24h). Small-RNA libraries
233 were generated using the mirVanaTM mircoRNA Isolation Kit
234 (Ambion, USA), according to the manufacturer's instructions.
235 Small-RNA libraries were prepared from three biological
236 replicates for each sample.
237 Total RNA was ligated with 3′ and 5′ RNA adaptors. Fragments
238 with adaptors on both ends were enriched by PCR after reverse
239 transcription, as described previously (Wang et al., 2017). The
240 resulting cDNAs were purified and enriched with 6% denaturing
241 polyacrylamide gel electrophoresis to isolate the fractions of the
242 expected size and to eliminate unincorporated primers, primer

dimer products, and dimerized adaptors (Wang et al., 2017). Finally, the nine resulting RNA libraries were sequenced using an Illumina/Solexa Genome Analyzer, at Guangzhou Genedenovo Biotech Company (Guangzhou, China).

Sequencing Data Analysis

As we described previously (Wang et Al., 2017), the raw sequence data were filtered to remove low quality reads and adaptor sequences. After adaptor trimming, reads of 16–35 nt in length were kept for further bioinformatic analysis. The remaining reads were mapped to the *C. carpio* genome with a tolerance of zero mismatches in the seed sequence using Bowtie (version 1.1.0). Sequences mapping to the genome were kept for further analysis. The reads mapped to the *C. carpio* genome were subsequently analysed to annotate rRNA, tRNA, snRNA, snoRNA, and non-coding RNA sequences by blasting against the Rfam (11.0, http://rfam.xfam.org) and GenBank (http://www.blast.nvbi.nlm.nih.gov/) databases. The remaining sequences were identified as the conserved miRNAs in carp by blasting against miRBase 21.0 allowing no more than two mismatches. Existing carp miRNAs referring to *C. carpio* miRNA were included in the miRBase with no base mismatch.

The sequences that did not match existing or conserved miRNAs were used to identify potentially novel miRNA candidates (Griffiths-Jones, 2006; Pearson, 1991). Novel miRNA candidates were identified by folding the flanking genome sequence of unique small RNAs using MIREAP (https://sourceforge.net/projects/mireap/). The enrichment level of each miRNA was identified by counting the number of reads in each sample. To identify differentially-expressed miRNAs within the nine libraries, the frequency of miRNA counts was normalized as transcripts per million (TPM). The TPM values were calculated as follows: normalized expression, TPM = (actual miRNA count/number of total clean reads) × 1,000,000. Only the miRNAs with over 2-fold changes in the two compared samples were considered differentially-expressed miRNAs ($P < 0.05$) (Audic et al., 1997). A positive value represents up-regulation of a miRNA, while a negative value indicates down-regulation.

Prediction of miRNA Targets

Target genes of miRNAs were predicted using RNAhybrid (v2.1.2) + svm light (v6.01), miRanda (v3.3a) and Targetscan software. The overlap of the predicted results from the three

programs was considered to represent the final result of predicted target mRNAs.

Gene Ontology (GO) and Pathway Analysis of Atrazine-Responsive mRNA Targets

Pathway analysis of the predicted target mRNAs was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/pathway.html) (Kanehisa et al., 2008). To classify the selected genes into groups with similar patterns of expression, each gene was assigned to an appropriate category, according to its main cellular function. To determine the biological phenomena target mRNAs were involved in, the DAVID (http://david.abcc.ncifcrf.gov/home.jsp) functional annotation clustering tool was used.

QPCR for Validation of miRNAs

The expression profiles of six randomly-selected miRNAs were investigated with qRT-PCR to validate their expression changes. Total RNA (500 ng) was converted to cDNA using miScript reverse transcriptase mix (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. QRT-PCR was

carried out using an Applied Biosystems 7300 Real-Time PCR System according to the standard protocol. CDNA samples were diluted to 1:150; 5 μL were used for each real-time PCR reaction. The 20-μL PCR mixture included 10 μL SYBR Premix Taq (2×), 0.4 μL miRNA-specific forward primers (10 μM), 0.4 μL miScript universal primer (10 μM), and 1 μL PCR template (cDNA). The PCR thermal program was 50 °C for 2 min, followed by 40 cycles of 95 °C for 2 min, 95 °C for 15 s, and 60 °C for 30 s. Melting curve analysis was performed after amplification. Standard curves for endogenous control and for all miRNAs were constructed using serial dilutions of a pooled cDNA sample. Standard curves were used to determine the quantity of the selected miRNAs and reference genes. Relative miRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was run in triplicate. SnRNA U6 was used as an endogenous control for QPCR of miRNAs.

RESULTS

Construction of cDNA Libraries for Sequencing and Small-RNA Discovery

We constructed nine cDNA libraries of small RNAs using pooled total RNAs from gonad tissues exposed to atrazine or

331 control tissues collected from primordial gonad (PG) and from
332 juvenile gonad stage carps. After filtering out low quality
333 sequences, 5′ and 3′ adapters, and reads < 18 nt, A total of
334 10,281,292, 10,086,295, 11,985,647, 10,080,133, 11,724,632,
335 11,604,659, 11,502,749, 11,030,073, and 11,282,882 clean reads
336 were obtained from the nine libraries. Solexa sequencing was
337 then performed for further analysis (Table 1). After comparing
338 the small-RNA sequences with NCBI GenBank and RFam, we
339 removed known types of RNA sequences including rRNA (3.84,
340 8.80, 8.84, 41.84, 16.31, 5.34, 0.85, 4.42 and 50.12%,
341 respectively), small nuclear RNA (snRNA), small nucleolar
342 RNA (snoRNA), and tRNA (2.16%; 1.84%; 1.08%; 0.47%;
343 1.08%; 2.72%; 0.58%; 2.79%; and 0.37%), and repeat
344 sequences. Because the genome of common carp is available,
345 the clean reads of small RNAs from the nine libraries were
346 mapped to the common carp genome with miRDeep2 software.
347 A total of 4,895,831 (82.84%); 4,657,428 (84.16%);
348 7,083,013(84.91%); 5,536,128 (90.12%); 6,104,227 (86.69%);
349 5,627,337 (82.83%); 5,334,147 (79.12%); 5,337,365 (79.15%)
350 and 7,463,759 (89.37%) of miRNA clean reads were mapped to
351 the common carp genome. The length distribution of the
352 high-quality reads had different trends in the samples within the

nine libraries. In the case of PG-CK samples, two peaks of length were observed at 22 nt and 27 nt. However, the size distribution of 21–23 nt increased and the size distribution of 26–29 nt decreased, after exposed to atrazine for 8 h and 24 h (Fig. 1). In the case of IIX-CK samples, higher miRNA mapped rates were observed in small RNAs of 26–28 nt in length. The size distribution of 21–23 nt increased and the size distribution of 26–29 nt decreased after exposure to atrazine for 8 h and 24 h (Fig. 1). In the case of IIC-CK samples, higher miRNA mapped rates were observed in small RNAs of 21–23 nt in length. The size distribution of 21–23 nt decreased and the size distribution of 27–29 nt increased after exposure to atrazine for 8 h and 24 h (Fig. 1). MiRNAs in small RNAs of 26–29 nt in length corresponded to Piwi-interacting RNAs (piRNAs) (Fig. 1), which are endogenous small non-coding RNA molecules 26–31 nt in length. Various studies have shown that Piwi–piRNA complexes are essential in gene silencing and in transposon regulation during germ cell differentiation and gonad development in animals (Klattenhoff and Theurkauf, 2008; Grentzinger et al., 2012; Kawaoka et al., 2012).

Identification of miRNAs

To identify miRNAs in the gonad of the Yellow River carp exposed or not to atrazine, the clean reads were used and the miRNAs identified by comparison to the deposited miRNAs from miRBase. Mireap_v0.2 software was used for secondary structure prediction of novel miRNA. There was a total of 4,443 miRNAs that were identified, including 3795 existing miRNAs, and 648 conserved miRNAs. Among the existing and conserved miRNAs, 7 miRNAs (ccr-miR-26a, ccr-miR-10b, ccr-miR-143, ccr-miR-181a, ccr-miR-100, ccr-miR-22a, and ccr-miR-92a) were the most abundant (TPM > 10,000) in all samples (TPM = Readout × 1,000,000 / Mapped reads).

Validation of miRNAs with qRT-PCR

To validate the results of Solexa sequencing, qRT-PCR was used to test six randomly-selected (ccr-miR-24, ccr-miR-146a, ccr-miR-192, ccr-miR-21, ccr-miR-143, and ccr-miR-454b) miRNAs. According to sequence analysis, from the miRNAs selected for comparison, three miRNAs (ccr-miR-146a, ccr-miR-21, and ccr-miR-454b) were up-regulated in juvenile ovary gonad at 24 h whereas three miRNAs (ccr-miR-24, ccr-miR-192, and ccr-miR-143) were down-regulated in juvenile ovary at 24 h of atrazine exposure. The relative expression levels of all six miRNAs were consistent with the sequencing

data (Fig. 2), indicating the reliability of the miRNA expression and correlation analysis based on small-RNA sequencing.

Effects of Atrazine Exposure on miRNA Expression in PG of Yellow River Carp

Primordial gonad is a crucial stage of sex differentiation, because of the formation of primordial germ cell. A comparative analysis of miRNA expression profiles with or without atrazine exposure may reveal miRNAs with important roles in early gonad differentiation. The results showed that atrazine exposure resulted in the altered expression of a larger number of miRNAs in PG compared with control. Atrazine exposure not only affected the total number of detectable miRNAs, but also the expression levels of miRNAs. After atrazine exposure for 8 h and 24 h, we observed different patterns of differentially-expressed miRNAs in PG of carp. Compared with the control group, 277 miRNAs were up-regulated and 334 miRNAs were down-regulated after atrazine exposure for 8 h. A significant difference in miRNA expression was observed between samples from atrazine exposure for 24 h and unexposed controls, 181 miRNAs were up-regulated and 1,056 miRNAs were down-regulated (Fig. 3). The most significantly

down-regulated miRNAs were miR-205, miR-184 and miR-203b-3p, which were down-regulated by 7.15, 3.61 and 3.35 fold, respectively. The most significantly up-regulated miRNAs were miR-7132, miR-135c, and miR-187 which were up-regulated by 8.70, 2.88 and 2.48 fold, respectively (Table 2). Atrazine exposure for 24 h had a greater effect on carp PG miRNA expression than the exposure for 8 h. The number of miRNAs with altered expression after atrazine exposure was higher at 24 h than at 8 h. However, the extent of change varied among the miRNAs. For example, the expression levels of miR-135c and miR-738 increased significantly (2.21- and 2.47-fold, respectively), whereas the expression levels of miR-203a decreased significantly (12.0-fold). Similarly, the changes in miRNA expression in PG varied between the unexposed control and atrazine exposure for 8 h or 24 h. For example, miR-135c was up-regulated by 2.2-fold after atrazine exposure for 8 h and was up-regulated by 2.8-fold after atrazine exposure for 24 h. MiR-122 was up-regulated by 1.4-fold after atrazine exposure for 8 h, but was down-regulated by 2.9-fold after atrazine exposure for 24 h. The miRNAs that were significantly altered in PG after exposure to atrazine may thus be involved in sex differentiation and development, and their

441 importance in sex differentiation mechanisms needs to be
442 clarified.
443
444 **Effects of Atrazine Exposure on miRNA Expression in**
445 **Juvenile Gonad of Yellow River Carp**

446 We observed patterns of differentially-expressed miRNAs in
447 juvenile gonad (stage II ovary and stage II testis) of carp after
448 atrazine exposure for 8 h and 24 h, especially in juvenile ovary
449 (Fig. 3). In juvenile ovary, 1053 miRNAs were up-regulated and
450 132 miRNAs were down-regulated after atrazine exposure for 8
451 h, relative to unexposed controls. Relative to the control group,
452 1085 miRNAs were up-regulated and 84 miRNAs were
453 down-regulated after atrazine exposure for 24 h. The most
454 significantly down-regulated miRNAs were miR-184, miR-214
455 and miR-122, which were down-regulated by 13.69, 13.21 and
456 12.40 fold respectively. The most significantly up-regulated
457 miRNAs were miR-17-3p, miR-454a, and miR-454b which
458 were up-regulated by 2.95, 2.49 and 2.42 fold respectively. In
459 juvenile testis, 561 miRNAs were up-regulated and 434
460 miRNAs were down-regulated after atrazine exposure for 8 h,
461 relative to the control group. Compared with the control group,
462 775 miRNAs were up-regulated and 799 miRNAs were

463 down-regulated after atrazine exposure for 24 h. The most
464 significantly down-regulated miRNAs were miR-205, miR-194,
465 and miR-122, which were down-regulated by 14.27, 13.59, and
466 11.81 fold, respectively. The most significantly up-regulated
467 miRNAs were miR-489, miR-738, and miR-193a, which were
468 up-regulated by 10.61, 4.53, and 2.50 fold, respectively.
469 Atrazine exposure for 24 h had a greater effect on juvenile testis
470 miRNA expression than 8 h exposure. In addition, atrazine
471 treatment led to a larger number of miRNAs with altered
472 expression in juvenile testis, than in juvenile ovary. The number
473 of down-regulated miRNAs was higher in juvenile testis than in
474 ovary which is consistent with the feminizing effects of atrazine.

475 The extent of expression change varied among miRNAs. For
476 example, in juvenile ovary, the miR-301a and miR-17-3p
477 expression levels decreased by 1.38- and 2.95-fold after atrazine
478 exposure for 24 h, respectively. In contrast, the miR-101b
479 expression level decreased by 1.01-fold. In juvenile testis, the
480 miR-193a and miR-146a expression levels increased by 2.50-
481 and 1.71-fold, respectively, after atrazine exposure for with 24 h.
482 In contrast, the miR-122 expression levels decreased by
483 11.81-fold. Similarly, the changes in miRNA expression of
484 juvenile ovary and testis varied between unexposed controls and

atrazine exposure for 8 h or 24 h. For example, ccr-miR-210 was down-regulated by 1.36-fold after atrazine exposure for 8 h, and was down-regulated by 2.06-fold after atrazine exposure for 24 h in juvenile ovary. Ccr-miR-192 was down-regulated by 3.12-fold after atrazine exposure for 8 h, and was down-regulated by 4.07-fold after atrazine exposure for 24 h (Table 2). In juvenile testis, ccr-miR-205 was down-regulated by 3.50-fold after atrazine exposure for 8 h but was down-regulated by 14.27-fold after atrazine exposure for 24 h (Table 2). The miRNAs that were significantly altered in juvenile gonad after exposure to atrazine may thus be involved in sex differentiation and development, and their importance in sex differentiation mechanisms needs to be clarified.

Expression Patterns of miRNAs at Different Gonad Developmental Stages in Yellow River Carp

Trend analysis of miRNA expression after exposure to atrazine for 8 h and 24 h, at different developmental stages, was conducted. In PG, we identified eight different expression patterns (Fig. 4), including 25 miRNAs that were up-regulated and 214 that were down-regulated during atrazine exposure (Fig. 4, profiles 3, 0). Expression of 232 miRNAs, such as miR-1 and

507 miR-133a-3p, increased after exposure for 8 h, but decreased at
508 24 h (Fig. 4, profile 5). In contrast, 129 miRNAs, including
509 miR-29a and miR-29b, showed the opposite expression pattern
510 during atrazine exposure (Fig. 4, profile 2). In juvenile ovary, 8
511 different expression patterns (Fig. 4) were identified, including
512 440 miRNAs that were up-regulated and 26 that were
513 down-regulated during atrazine exposure (Fig. 4, profiles 7, 0).
514 Expression of 157 miRNAs, such as mir-202-y and mir-27c-5p,
515 increased after exposed for 8 h, but decreased at 24 h (Fig. 4,
516 profile 5). In contrast, 70 miRNAs, including miR-155 and
517 miR-92b, showed the opposite expression pattern during
518 atrazine exposure (Fig. 4, profile 2). In juvenile testis, we also
519 identified eight different expression patterns (Fig. 4), including
520 68 miRNAs that were up-regulated and 73 that were
521 down-regulated during exposure (Fig. 4, profiles 7, 0).
522 Expression of 117 miRNAs, such as mir-15a and mir-16a,
523 increased after atrazine exposure for 8 h, but decreased at 24 h
524 (Fig. 4, profile 5). In contrast, 41 miRNAs, including miR-144
525 and miR-148, showed the opposite expression pattern during
526 atrazine exposure (Fig. 4, profile 2).

527 In this study, miRNAs targeting male-biased genes showed an
528 upward trend. In PG, miR-499, which was predicted to target

529 *sox9*, increased after exposure to atrazine (Fig. 4, profile 7).
530 *Gsdf* was the predicted target of miR-146a and of miR-22a,
531 which also increased after exposure to atrazine (Fig. 4 profile 7).
532 The expression profiles of miR-72-x and miR-212-y, which
533 were predicted to target *dmrt*, were also consistent with the
534 above miRNAs which predicted male-biased target genes (Fig. 4,
535 profile 7). In juvenile ovary, novel-m3245-5p, which was
536 predicted to target *sox9*, increased after exposure to atrazine (Fig.
537 4, profile 7). *Gsdf* was the predicted target of novel-m0192-3p
538 and novel-m0514-3p, which also increased after exposure to
539 atrazine (Fig. 4, profile 7). MiR-454a and miR-454b, which
540 were predicted to target *atm*, increased after exposure to atrazine
541 (Fig. 4, profile 7). The expression profiles of novel-m0515-3p
542 and novel-m0080-5p, which were predicted to target *dmrt*, were
543 also consistent with the above miRNAs which predicted
544 male-biased target genes (Fig. 4, profile 18). In juvenile testis,
545 novel-m3312-3p, which is predicted to target *sox9*, increased
546 after exposure to atrazine (Fig. 4, profile 7). *Atm*, which was the
547 predicted target of novel-m0167-3p and novel-m0417-3p, also
548 increased after exposure to atrazine (Fig. 4, profile 7).

549 In contrast, miRNAs targeting female-biased genes showed a
550 downward trend. Expression levels of novel-m0101-3p and

551 novel-m3450-3p in PG, miR-101b in juvenile ovary, and
552 miR-203b-3p in juvenile testis, all of which were predicted to
553 target *Smad4*, decreased after exposure to atrazine (Fig. 4,
554 profile 0). The most abundant differentially-expressed miRNAs
555 after exposure to atrazine in PG, juvenile ovary and juvenile
556 testis were let-7a, miR-143, and miR-125b, all of which
557 decreased significantly during atrazine exposure.
558 These results suggest that these miRNAs may influence gonad
559 development.
560
561 **Identification and Signalling Analysis of Target Genes of**
562 **Differentially-Expressed miRNA**
563 To identify potential targets of differentially-expressed miRNAs,
564 involved in sex differentiation and development after atrazine
565 exposure, we performed target-gene prediction based on the
566 common carp (*C. carpio*) genome sequence
567 (http://www.carpbase.org/). A total of 26,299 genes were
568 predicted to be the possible targets of 4353
569 differentially-expressed miRNAs that were commonly
570 expressed in all atrazine exposure samples. Functional
571 annotation using KEGG identified 239 annotated signalling
572 pathways, including at least 11 pathways involved in

573 reproductive biology: transforming growth factor-β (*TGF-β*)
574 signalling, *Wnt* signalling, oocyte meiosis, mitogen-activated
575 protein kinase (*MAPK*) signalling, Notch signalling, p53
576 signalling, gonadotropin-releasing hormone (*GnRH*) signalling,
577 RNA polymerase, steroid hormone biosynthesis, estrogen
578 signalling pathway, and metabolism of xenobiotics by
579 cytochrome P450. Interestingly, the target genes of 790
580 miRNAs belonged to the *MAPK* signalling pathway, which
581 plays an important part in virtually every step of
582 spermatogenesis in the testis. The *MAPK* signalling pathway is
583 also involved in the acrosome reaction in the female
584 reproductive tract before fertilization of the ovum (Huang et al.,
585 2011). *Wnt* signalling is known to be involved in mammalian
586 reproduction (Kobayashi et al., 2011), and in zebrafish sex
587 determination (Chang et al., 2013). We detected 415 miRNA
588 targets belonging to the *Wnt* signalling pathway, 30 belonging to
589 *NF-kappa B* signalling pathway, and 133 belonging to p53
590 signalling pathway. *Wnt* signalling pathway, *NF-kappa B*
591 signalling pathway and p53 signalling pathways were associated
592 with sex differentiation in zebrafish (Chang et al., 2013). Target
593 genes predicted to belong to the three pathways in our study
594 may be involved in sex differentiation and gonad development

595 in Yellow River carp. Moreover, we identified 245 miRNA
596 targets belonging to the *TGF-β* signalling pathway, and 179
597 belonging to the Notch signalling pathway. In addition, we also
598 identified 31 miRNA targets belonging to oestrogen signalling
599 pathway, which may play an important role in hormone
600 regulation.

601 To determine the key biological process of the putative target
602 genes related to atrazine exposure, GO analysis was performed.
603 The identified biological processes that the putative target genes
604 were classified into include reproduction, reproductive process,
605 response to stimulus, developmental process, and growth, which
606 were all mechanisms related to sex differentiation and gonad
607 development. The results showed possible relationships between
608 atrazine, putative targets and gonad development, and suggested
609 that atrazine may have effect on sex differentiation and gonad
610 development.

611 We analysed the relationships between
612 differentially-expressed miRNAs and their putative target genes.
613 *Foxl2*, *stat1*, *sf1*, *dmrt* and *gsdf* have been shown to be key
614 factors in early ovary differentiation (Ijiri et al., 2008;
615 Nagahama et al., 1997). We also analysed *smad3*, *smad4*, *sox9*,
616 and *atm*, which are also known to be responsible for gonad

differentiation. We found that these genes were predicted targets of many miRNAs, which could thus negatively regulate these target genes. Given the important roles of steroid hormones in reproduction and sexual dimorphism in fish, we analysed the relationships between miRNAs, including *hsd11b* and *hsd3b* (which encode key enzymes in the steroid hormone biosynthesis pathway), mRNAs, and the steroid hormone biosynthesis pathway. Atrazine has endocrine-disrupting effects by altering the HPG axis (Trentacoste et al., 2001). We analysed genes that have critical roles in the regulation of the HPG axis including *ER1*, *ER2*, *AR*, and *CYP19A1*.

In the PG, a higher number of miRNAs targeting female-biased genes were down-regulated. MiR-135c which was significantly down-regulated by 2.21-fold and 2.88-fold after exposure to atrazine for 8 h and 24 h, respectively, were predicted to target *ER*, *foxl2*, and *CYP19A*. *Gsdf* was the predicted target of miR-132a, miR-146a, miR-210, and miR-22a which were also down-regulated. Our results indicated that atrazine can promote early gonad-determining genes by down-regulating miRNAs. miR-205, which was predicted to target *atm*, *EGF*, *bcl2*, *BMP1* (bone morphogenetic protein 1), was significantly up-regulated by 7.23-fold for 8 h and 7.15-fold

639 for 24 h, respectively. miR-132a, which targeted *dmrt2*, was
640 up-regulated by 1.22-fold after exposure to atrazine for 24 h, but
641 it was not at 8 h. miR-499, which also targeted *dmrt2*, was
642 up-regulated by 1.46-fold for 8 h and 1.57-fold for 24 h,
643 respectively. After exposure to atrazine for 24 h, miR-202x, and
644 miR-374-y, which were predicted to target *smad3*, were
645 up-regulated and down-regulated, respectively. *Hsd11b* was
646 predicted to target miR-216-x and miR-342-y. *Hsd3b* was the
647 predicted target of let-7-z. *Stat1* was predicted to be the target of
648 miR-135c and miR-430, *Sf1* of miR-154-y and miR-3958-y, and
649 *Sox9* of miR-499. These results illustrate the possible roles of
650 the differentially-expressed miRNAs in PG, after exposure to
651 atrazine during gonad differentiation.

652 In juvenile ovary, miR-21, which was significantly
653 up-regulated by 2.18-fold after exposure to atrazine for 24 h,
654 was predicted to target *AR* and *atm*. MiR-101b, which was
655 predicted to target *sf1*, was significantly down-regulated by
656 1.01-fold. MiR-132a, which was significantly up-regulated by
657 1.08-fold after exposure to atrazine for 8 h, was predicted to
658 target *AR*, *dmrt2*, *gsdf*, and *atm*. *Smad4* was predicted to target
659 novel-m0048-5p. *Hsd11b* was predicted to target
660 novel-m0305-3p. *Hsd3b* was the predicted target of miR-410-x.

661 *Stat1* was predicted to be the target of miR-192, *CYP19A* of
662 miR-203a and novel-m0527-3p, and *Sox9* of novel-m0011-5p.
663 In juvenile testis, miR-181b, and miR-181c, which were
664 significantly up-regulated by 1.04-fold and 1.41-fold,
665 respectively, after exposure to atrazine for 24 h, were predicted
666 to target *dmrt2* and *atm*. miR-146a, which was predicted to
667 target *gsdf* was significantly up-regulated by 1.70-fold.
668 MiR-132a, which was significantly down-regulated by 1.28-fold,
669 was predicted to target *ER*. *Smad4* was predicted to target
670 miR-200b. *Hsd11b* was predicted to target novel-m0305-3p.
671 *Hsd3b* was the predicted target of miR-410-x. *Stat1* was
672 predicted to be the target of miR-192, *CYP19A* of miR-203b-3p
673 and novel-m0693-5p, and *Sox9* of novel-m0081-5p. These
674 results indicate that atrazine promotes the biosynthesis of steroid
675 hormone by altering the miRNAs.
676 These differentially-expressed miRNAs were also predicted
677 to be involved in many reproductive biology pathways,
678 including steroid metabolic processes, *TGF-β* receptor
679 signalling, *Wnt* signalling, and cell differentiation. Moreover
680 after exposure to atrazine for 24 h, the predicted target genes of
681 the differentially-expressed miRNAs of PG included *cyp51a1*,
682 *hsd3*, *smad4*, *lemd3*, *zranb1*, *tbx6*, *grk6*, *ccna1*, *pcna*, *GATA*,

683 *RBMS1* and *prosapip1*, and some of which, such as *cyp51a1* and
684 *hsd3*, are gonad development-related genes. Many other
685 miRNAs were also predicted to target genes associated with
686 reproductive processes. miR-205 and miR-135c were predicted
687 to target *bcl2* and *notch2*, which belong to the *TGF-β* signalling
688 and Notch signalling pathways, respectively. miR-205 was
689 predicted to target *pdk1* and *inhibin beta A chain*, which are
690 related to *TGF-β* signalling, and female gonad development,
691 respectively. Although the predicted target genes need to be
692 validated experimentally, these results illustrate some of the
693 possible roles of the differentially-expressed miRNAs in gonad
694 reproductive processes.
695

696 **DISCUSSION**

697 MiRNAs are involved in diverse biogenesis pathways and have
698 versatile regulatory functions in differentiation, proliferation,
699 and apoptosis (Bartel 2009). To date only a limited number of
700 studies have investigated miRNA expression alterations in
701 response to exposure to endocrine-disrupting chemical in fish
702 and humans (Avissar-Whiting et al., 2010; Hsu et al., 2009;
703 Jenny et al., 2012; Tilghman et al., 2012; Veiga-Lopez et al.,
704 2013). There are only few reports on the miRNA profiling of

fish, in response to atrazine exposure, and no reports in common carp. The investigation into the adverse effects of atrazine exposure on miRNAs is important to reveal the molecular mechanism of gonad differentiation. In the present study, we assessed the potential effects of atrazine on miRNAs in the reproductive system at two developmental stages (PG and II-stage gonad) of Yellow River carp. Primordial germ cell formation is a crucial stage of gonad differentiation, and II-stage gonad is the stage of evident sex differentiation. Comparative analysis of miRNA expression profiles at these two important stages, after exposure to atrazine, is helpful to identify miRNAs that play important roles in gonad differentiation.

In this study, atrazine exposure resulted in significant expression alterations of various miRNAs. Atrazine exposure for 24 h caused more alterations in the expression of miRNAs than exposure for 8 h. Atrazine exposure for 24 h caused more alteration in miRNA expression in juvenile testis than in juvenile ovary. It is thus clear that acute and short-time exposure to atrazine during development can produce adverse effects, as has been suggested before (Kathryn et al., 2016).

Several studies in amphibians have suggested that atrazine is associated with feminization of males in the wild (Hayes et al.,

727 2002; Hayes et al., 2002; Murphy et al., 2006). In field studies,
728 atrazine has repeatedly been associated with the presence of
729 feminized secondary sex characteristics in male frogs (McCoy et
730 al., 2008). In fish, atrazine causes degeneration of interstitial
731 tissue in the testes (Spano et al., 2004) and feminizes the gonads
732 of developing male teleost fish (Tillitt et al., 2008). In addition,
733 embryonic atrazine exposure alters the expression of zebrafish
734 and human miRNAs known to play a role in angiogenesis,
735 cancer, neuronal development, differentiation, and maturation
736 (Sara et.al 2016). In our study, atrazine exposure altered the
737 expression of carp miRNAs that play a role in gonad
738 differentiation and gonad development. A number of miRNAs
739 (including miR-122, let-7, miR-192, miR-21, miR-499,
740 miR-146, miR-101, miR-128, and miR-124) that are highly
741 expressed in adult bighead carp and silver carp were
742 significantly altered in our study (Chi et al., 2011).
743 Our results suggest that miR-21, let-7, miR-430, miR-181a,
744 and miR- 143 may play important roles in gonad differentiation
745 and development in Yellow River carp.
746 Several studies suggested that miR-21 may play an important
747 role in gonad development. A study reported that in cattle
748 miR-21 was significantly up-regulated in the ovary (relative to

749 testis) suggesting that miR-21 may play a regulatory role in
750 female physiology (McBride, 2012). A previous study indicated
751 that miR-21 plays a role in preventing apoptosis in periovulatory
752 granulosa cells, as they transit into luteal cells (Christenson et al.,
753 2010). Has-miR-21 was also up-regulated by ovarian steroids in
754 mouse granulosa cells and human endometrial stromal cells, and
755 in glandular epithelial cells (Fiedler et al., 2008; Pan et al.,
756 2007). In this study, atrazine exposure did not change the
757 expression of miR-21 in the PG after atrazine exposure, but
758 induced its up-regulation in juvenile ovary and down-regulation
759 in juvenile testis.

760 The predicted target genes of miR-21 included genes of the
761 *MAPK*, B-cell receptor, *TGF-β*, and apoptotic pathways. This
762 observation suggests that miR-21 may play crucial roles in
763 ovary development, gonad differentiation (Gangaraju and Lin et
764 al., 2009), and endocrine regulation (Eshel et al., 2014; Huang et
765 al., 2011). The predicted target genes of miR-21 in our study
766 were *AR* and *atm*.

767 Let-7 was another family of miRNAs with altered expression
768 by atrazine exposure. The let-7 family was first discovered and
769 characterized in *Caenorhabditis elegans*, and plays an important
770 role in regulating late developmental events by down-regulating

771 lin-41, and possibly other genes (Pasquinelli et al., 2000). Let-7
772 was significantly up-regulated after atrazine exposure in the PG
773 and juvenile testis. The predicted target genes of let-7 in our
774 study were *sox9* and *atm*.

775 The miR-430 family is known to be involved in embryonic
776 morphogenesis and clearance of maternal mRNAs; it is and
777 highly expressed during early zebrafish development (Choi et al.,
778 2007; Giraldez et al., 2005; Giraldez et al., 2006; Inui et al.,
779 2010). MiR-430 has been shown to target chemokine signalling
780 to ensure accurate migration of primordial germ cells (Staton et
781 al., 2011). In our study and miR-430 was down-regulated in PG
782 but not in juvenile gonad, which indicates that miR-430 has an
783 important role in early gonad differentiation of Yellow River
784 carp.

785 Several reports showed that miR-143 is highly expressed in
786 the juvenile ovary; it is a dominant miRNA in ovaries in cattle,
787 pigs, and yellow catfish (Li et al., 2009; Lau et al., 2014). In this
788 study, miR-143 was highly expressed in juvenile ovary, which is
789 in keeping with previous reports.

790 The miR-181a family is abundantly expressed in the gonads
791 of tilapia (Hossain et al., 2012), mice (Saunders et al., 2010),
792 and humans (Sirotkin et al., 2009). It was down-regulated in

793 juvenile ovary in the present study. Overall, above results
794 suggest that miR-21, let-7, miR-430, miR-181a, and miR- 143
795 may play important roles in goand differentiation and
796 development in Yellow River carp.

797 Differentially expressed miRNAs showed a variety of
798 expression patterns at different development stages. Among the
799 8 different expression patterns, two patterns are particularly
800 worthy of attention, involving miRNAs with expression levels
801 that either increased or decreased significantly after atrazine
802 exposure. MiRNAs whose expression either increased or
803 decreased significantly after atrazine exposure may be direct
804 regulators of gonad differentiation. Samples with the highest
805 number of miRNAs with altered expression were the PG and
806 juvenile ovary exposed to atrazine for 8 h or 24 h. The number
807 of decreased miRNAs was 1,056 in PG, including miRNAs
808 which targets were female-biased. Because miRNAs are
809 negatively correlated with its target genes, this observation
810 suggests that atrazine promotes the expression of female-biased
811 genes by decreasing specific miRNAs in PG, which would result
812 in the differentiation of the gonad to the female phenotype. The
813 juvenile ovaries exposed to atrazine had the highest number of
814 up-regulated miRNAs, including miRNAs whose targets are

815 male-biased. It is thus possible that atrazine represses the
816 expression of male-biased genes by increasing specific miRNAs
817 in juvenile ovary.

818 The juvenile testis exposed to atrazine had the highest number
819 of miRNAs with altered expression, indicating that this tissue
820 was more sensitive to atrazine, possibly leading to the
821 feminization of males. This observation suggests that these
822 miRNAs may have an important function in the timing of gonad
823 differentiation and development.

824 Target-gene prediction showed that many of the genes that we
825 identified as targets of the miRNAs that we studied were
826 involved in sex differentiation. Among these predicted genes,
827 *sox9*, *dmrt*, and *gsdf* have been identified as sex-determining
828 genes in fish (Diego et al., 2015; Myosho et al., 2012). For
829 example, *Hsd11b* and *hsd3b* encode key enzymes in the steroid
830 hormone biosynthesis pathway. These genes may participate in
831 steroid hormone synthesis, gonad function, and mechanisms of
832 sex differentiation, and may play a vital role in developmental
833 timing. However, further studies are needed to confirm the
834 interactions and functions of miRNA and target genes. In
835 addition, the results also show that atrazine has oestrogenic
836 effects down-regulating male-biased genes (such as *dmrt* and

837 *atm*) through specific miRNAs up-regulation, and up-regulating
838 female-biased genes (such as *foxl2*) through specific miRNAs
839 down-regulation.

840 Previous studies showed that atrazine exposure can
841 significantly reduce synthesis, secretion, and the circulating
842 levels of androgens in fish (Moore et al., 1998; Spano et al.,
843 2004), amphibians (Hayes et al., 2002; Hayes et al., 2010),
844 reptiles (Rey et al., 2009), and mammals (Friedmann et al., 2002;
845 Stoker et al., 2000), and also in birds to a lower extent
846 (Wilhelms et al., 2006). The endocrine-disrupting effects of
847 atrazine are primarily due to alterations of the HPG axis (Cooper
848 et al., 2000; Foradori et al., 2009, 2013; Weber et al., 2013;
849 Wirbisky et al., 2016a). However, atrazine's mechanism of
850 action is not well-understood, it has been proposed that atrazine
851 up-regulate aromatase expression (Caron-Beaudoin et al., 2016;
852 Sanderson et al., 2000, 2001, 2002). Aromatase up-regulation
853 leads to increased conversion of androgens into oestrogens
854 (Laville et al., 2006). In the present study, we analysed genes
855 that regulate hormone biosynthesis in the HPG axis, including
856 *ER1*, *ER2*, *AR*, and *CYP19A1*. MiR-122, which targets *ER1* and
857 *ER2*, was down-regulated by atrazine. MiR-21, which targets *AR*
858 was up-regulated in PG by atrazine. MiR-203a, which targets

859 *CYP19A1*, was down-regulated in PG by atrazine. Our results
860 indicate that atrazine can up-regulate aromatase expression
861 through specific miRNAs, which is consistent with previous
862 studies.

863 We tested the hypothesis that atrazine has
864 endocrine-disrupting effects by altering genes of the HPG axis
865 through its corresponding miRNAs. In the PG, atrazine affects
866 sex differentiation mainly through altering upstream genes
867 involved in gonad differentiation. In juvenile ovary or testis,
868 atrazine affects the gonad development mainly through altering
869 hormone generation and the expression of hormone receptor
870 genes. Further studies are needed to investigate the mechanisms
871 and roles of miRNAs in the regulation of genes during gonad
872 differentiation and development.

873 In summary, atrazine exposure caused significant alterations
874 in miRNAs expression at the crucial stages of carp gonad
875 development. Target genes of differentially-expressed miRNAs
876 are key factors in early ovary differentiation or play an
877 important role in the formation of germ cells. In addition, our
878 results indicate that atrazine up-regulates aromatase expression
879 through specific miRNAs, supporting the hypothesis that
880 atrazine has endocrine-disrupting effects, altering the expression

of genes of the HPG axis through its corresponding miRNAs.

COMPETING INTEREST

The authors have declared that no competing interests exist.

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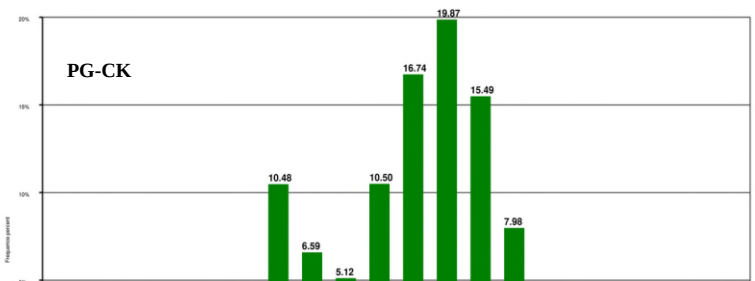
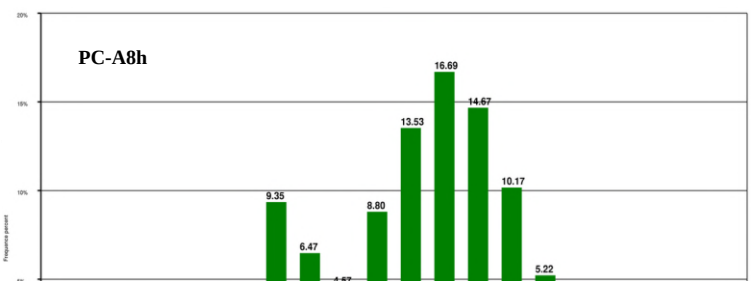
TABLE I. Distribution of sequenced clean reads

Type	PG-CK		PG-A8h		PG-A24h		IIC-CK		IIC-A8h	
	Total reads	%	Total reads	%	Total reads	%	Total reads	%	Total reads	%
tRNA	127738	2.16	102015	1.84	90495	1.08	28725	0.47	76379	1.08
snoRNA	491	0.01	1870	0.03	3233	0.04	1316	0.02	839	0.01
rRNA	226731	3.84	486980	8.80	737066	8.84	2569896	41.84	1148476	16.31
snRNA	3487	0.06	10178	0.18	15628	0.19	2201	0.04	1516	0.02
Clean reads	10281292	100	10086295	100	11985647	100	10080133	100	11724632	100

Type	IIC-A24h		IIX-CK		IIX-A8h		IIX-A24h	
	Total reads	%	Total reads	%	Total reads	%	Total reads	%
tRNA	184690	2.72	38943	0.58	188272	2.79	30837	0.37
snoRNA	206	0.00	161	0.00	3072	0.05	8262	0.10
rRNA	362576	5.34	57360	0.85	297759	4.42	4185775	50.12
snRNA	4984	0.07	611	0.01	10599	0.16	32132	0.38
Clean reads	11604659	100	11502749	100	11030073	100	11282882	100

Fig. 1. Length distribution of miRNA sequences from Yellow River carp in primordial gonad control (PG-CK), primordial gonad exposed to atrazine for 8 h (PG-A8h), primordial gonad exposed to atrazine for 24 h (PG-A24h), juvenile ovary control (IIC-CK), juvenile ovary exposed to atrazine for 8 h (IIC-A8h), juvenile ovary exposed to atrazine for 24 h (IIC-A24h), juvenile testis control (IIX-CK), juvenile testis exposed to atrazine for 8 h (IIX-A8h), juvenile testis exposed to atrazine for 24 h (IIX-A24h).





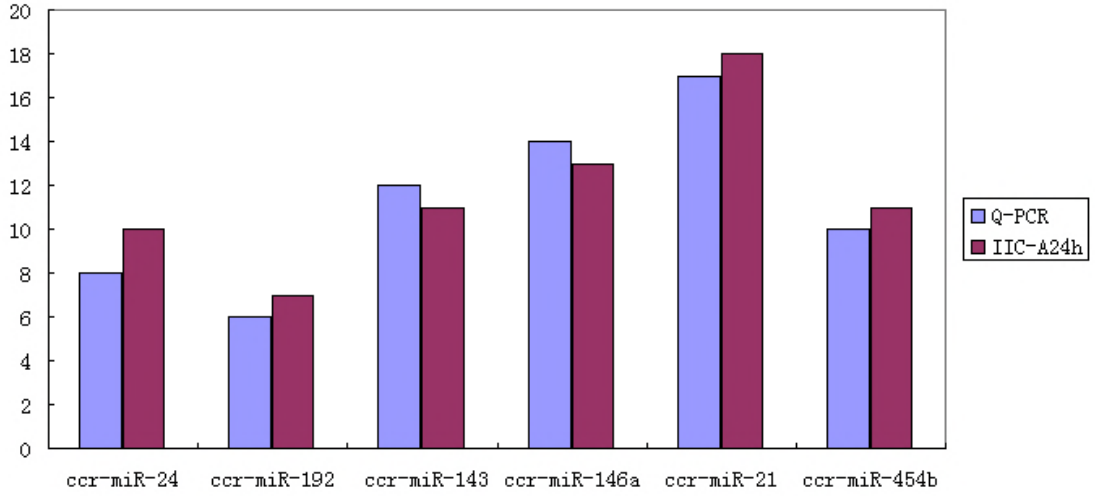


Fig. 2. Real-time quantitative PCR gene expression analysis of six randomly-selected miRNAs. Gene expression was normalized to the level of U6 snRNA.

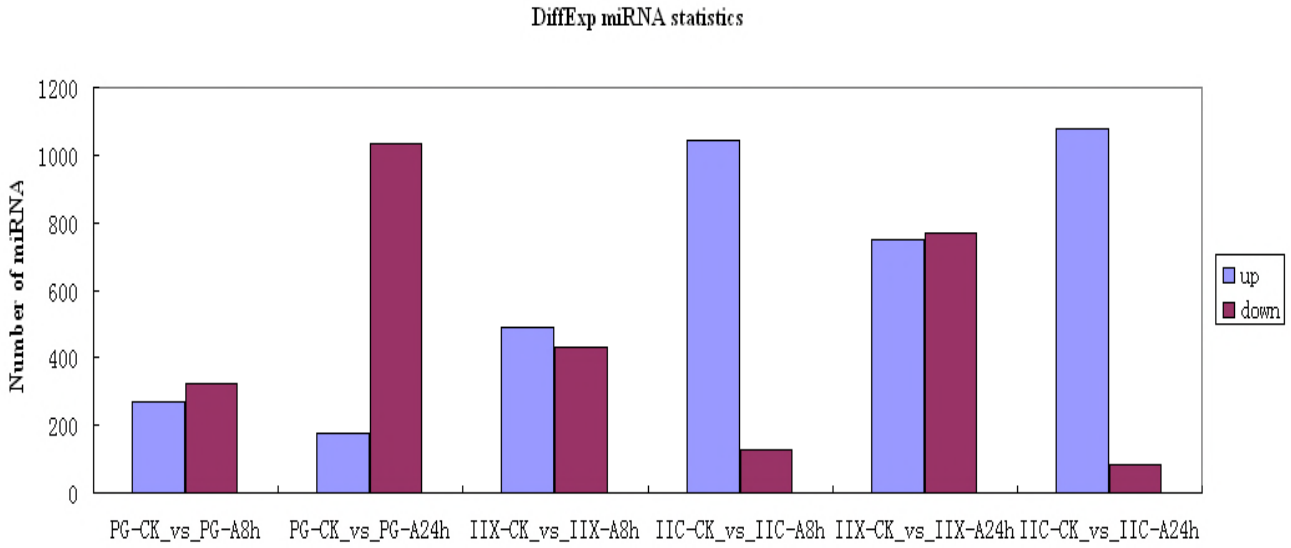


Fig. 3. Differential expression of miRNAs in Yellow River carp. Greater than 2-fold change while $P < 0.05$

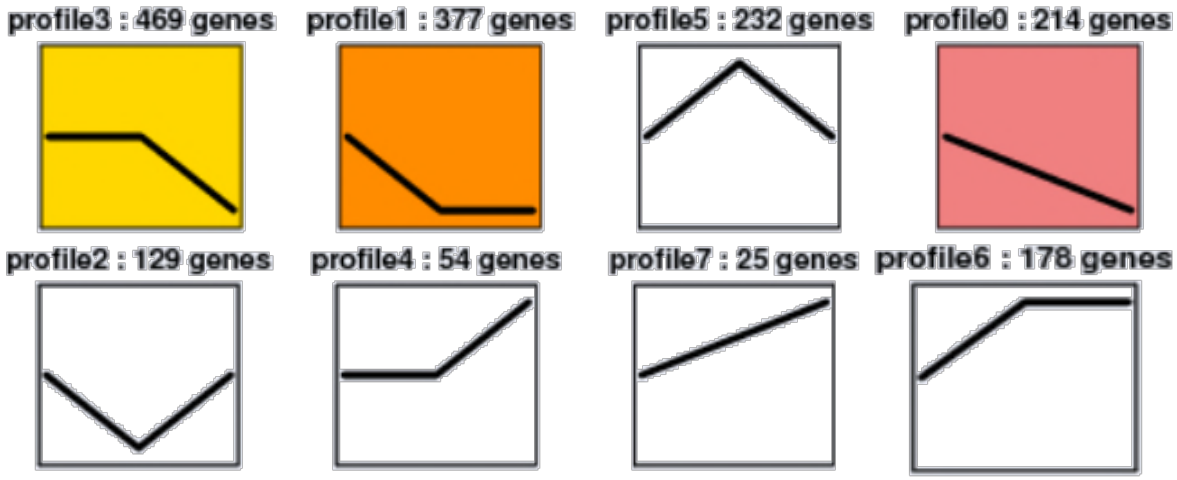


Fig. 4. Trend analysis of miRNA expression profiles after exposure to atrazine in Yellow River Carp

TABLE 2. miRNAs with significant expression alterations after atrazine exposure in Yellow River Carp

Name	Sample	Up or Down	log2(FC)
ccr-let-7a	PG-A24h	down-regulation	-1.36
ccr-miR-135c	PG-A24h	up-regulation	2.88
ccr-miR-122	PG-A8h	up-regulation	1.48
ccr-miR-192	IIC-A8h	down-regulation	-3.13
ccr-miR-146a	IIC-A24h	up-regulation	2.13
ccr-miR-184	IIC-A24h	down-regulation	-13.70
ccr-let-7b	IIX-A8h	up-regulation	1.17
ccr-miR-101a	IIX-A24h	down-regulation	-1.34
ccr-miR-205	IIX-A24h	down-regulation	-14.27
ccr-miR-193a	IIX-A24h	up-regulation	2.51