Fish Physiol Biochem (2019) 45:1409–1417
https://doi.org/10.1007/s10695-019-00643-4

Heat stress–induced alterations in the expression of genes associated with gonadal integrity of the teleost *Puntius sophore*

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Received: 21 May 2018 / Accepted: 22 April 2019 / Published online: 29 May 2019

Abstract Temperature plays an important role on reproductive physiology of vertebrates including mammals, fish, and birds. It has varying effects on fish reproduction depending on the species; higher temperatures favor the spring-spawning species, while lower temperatures stimulate reproduction in autumn spawners. To evaluate the impact of high temperature on the reproductive physiology of minnow *Puntius sophore*, we carried out expression analysis of selected genes associated with gamete quality (*hsp60*, *hsp70*, *hsp90*, *hsf1*, *vtg*), pleuripotency (*sox2*, *oct4*, *nanog*), and sex determination (*dmrt1*) in gonads (ovary and testis) of *P. sophore*, heat stressed for different time periods (36 °C/7 days or 60 days) using real-time quantitative polymerase chain reaction (RT-qPCR). Expression of most of the *hsp*, *vtg*, and pleuripotency marker genes *sox-2*, *oct-4*, and *nanog* genes was downregulated in both ovary and testis of heat-stressed fish. The expression of *dmrt-1* was upregulated in testis but downregulated in ovary of the heat-stressed fish which could be a male favoring effect of high temperature in *P. sophore*. This study suggests that the reproductive physiology and health of the nutrient dense *P. sophore* would be negatively affected by high temperature stress.

Keywords Temperature stress · Reproductive physiology · Heat-shock proteins · Pleuripotency marker genes · *Puntius sophore*

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Introduction

Temperature plays an important role in reproductive physiology of vertebrates including mammals, fish, and birds (Hansen 2009; Boulangé-Lecomte et al. 2014; Sengar et al. 2017). In mammals, high temperature disrupts spermatogenesis and oocyte development, oocyte maturation, early embryonic development, and foetal and placental growth and lactation (Hansen 2009; Boulangé-Lecomte et al. 2014). High temperature also affects reproductive cycles in birds; increase in environmental temperature has resulted in shifting of the egg-laying timings (Visser et al. 2009). Similarly, in case of fish, temperature has varied effects on reproduction, depending upon the species; higher temperature favor

the spring-spawning species, while lower temperature stimulates reproduction in autumn spawners (Pankhurst and Munday 2011). High temperature stress is the major environmental concern in the climate change regime, and assessing its impact on reproductive physiology of fish species is important as selection of species that can withstand higher temperatures with relatively lower impact on their reproductive performance would be required for sustainable aquaculture (Mohanty et al. 2010).

A number of experimental studies have shown that increase in temperature could affect reproduction in fish; however, the nature of these effects would depend on the period and amplitude and would vary from species to species (Pankhurst and King 2010; Pankhurst and Munday 2011). *Puntius sophore* is a micronutrient dense fish with a wide distribution in the tropical region, and it is being seen as an important tool for fighting hidden hunger and malnutrition (Mahanty et al. 2014). Thus, efforts are being made by aquaculturists to standardize its breeding and increase its production through aquaculture (Mohanty et al. 2018a, b). The breeding biology of *Puntius* has been reported earlier, and it has been seen that the fish is a seasonal breeder; the spawning period is marked by the gradual increase in the gonado-somatic index (GSI) during the month of March and July which then gradually starts decreasing (Choudhury et al. 2015; Hasan et al. 2018).

In this backdrop, the present study was carried out to investigate the impact of short-term (7 days) and long-term (60 days) high temperature stress on the reproductive physiology of this important species through expression analysis of a number of *hsp* genes (*hsp90*, *hsp70*, *hsp60*), heat-shock factor 1 (*hsf1*), vitellogenin (*vtg*), pleuripotency marker genes (*sox-2*, *oct-4* and *nanog*), and *dmrt-1*. Expression of heat-shock protein (*hsp*) and vitellogenin (*vtg*) genes were carried out as there are numerous reports which depict the important roles played by these genes in maintenance of gonadal integrity. Besides, *hsp70* has also been reported as a possible marker for assessing the egg quality (Kohn et al. 2015; Sullivan et al. 2015). Like the *hsps*, the pleuripotency markers, SRY (sex-determining region Y) -box 2 (*sox-2*), *octamer-binding transcription factor 4* (*oct-4*), and *nanog* are also considered as markers for assessing the quality of oocyte (Zuccotti et al. 2011).

Materials and methods

Ethics statement

The study including sample collection, experimentation, and sacrifice met the ethical guidelines including adherence to the legal requirements of the study country. The study was approved by the Institute Animal Ethics Committee (IAEC) of Central Inland Fisheries Research Institute vide approval no. CIFRI/IAEC-17/03.

Sample collection after short- and long-term thermal exposure

P. sophore (weight 4.22 ± 0.5 g, length 6.46 ± 0.56 cm) were collected from aquaculture ponds and were acclimatized under laboratory conditions for 30 days in acrylic tanks of 30 l capacity fitted with thermostats. Fishes were fed once daily by providing laboratory prepared feed. Feed was prepared using soybean oil cake (290 g Kg^{-1}), mustard oil cake (524 g Kg^{-1}), fish meal (50 g Kg^{-1}), vitamin–mineral premix (20 g Kg^{-1}), and edible vegetable oil (15 g Kg^{-1}). The protein and fat contents of the feed were 34% and 5.8%, respectively. *P. sophore* is found in almost all freshwater ecosystems in the tropical countries where the water temperature varies between 25 and 30 °C baring the peak summers. Therefore, the fishes were acclimatized at water temperature of 27 ± 0.2 °C prior to the heat-stress treatment. The fishes were randomly assigned to three experimental groups; one of the group maintained at 27 °C during the experimental regime served as the control. In the other two groups, the temperature was raised from 27 °C at the rate of 2 °C/h using a thermostat and temperature was maintained at 36 °C for 7 days and 60 days, respectively. A photoperiod of 12 h light and 12 h dark was maintained throughout the experimental regime. The water temperature was monitored using a calibrated thermometer.

The critical thermal maximum temperature (CTMax) of *P. sophore* has been found to be ranging between 39 and 41.5 °C depending upon the acclimatization temperature and other factors (Mahanty et al. 2016a, b, 2017). Therefore, a sublethal temperature close to the CTMax of the fish (36 °C) was chosen for this experimental study.

P. sophore is a prolific breeder, and its breeding biology has been studied by a number of researchers (Choudhury et al. 2015; Hasan et al. 2018). It has been

reported that the fish spawns during the months March to July (Choudhury et al. 2015; Hasan et al. 2018). In the present study, gonads of mature fishes were collected during the pre-spawning period after completion of the exposure period. Nine male and 9 female fishes from each group were euthanized with tricaine, MS-222, 200 mg/ml, prior to dissection and collection of tissues in RNA later (R0901, Sigma).

RNA extraction and cDNA synthesis

RNA was extracted from tissue samples (nine samples from each experimental group, weighing approximately 50–70 mg of tissues) using RiboZol (HiMedia, India) according to the manufacturer's protocol. RNA integrity was confirmed by determining their RNA integrity no. (RIN) by a Bioanalyzer (Agilent 2100), and samples with RIN values > 6 were processed for further analysis. RNA samples were treated with the DNase1 (NEB, UK) as per the manufacturer's recommended protocol to remove DNA contamination. One microgram of DNase treated total RNA was reversely transcribed using M-MLV reverse transcriptase (New England Biology, UK) according to manufacturer's protocols.

Primer synthesis

Expression analysis of four *hsp* genes: *hsp90*, *hsp70*, *hsp60*, and *hsf1*; three pleuripotency marker genes: *oct-4*, *nanog*, and *sox-2* were carried out in both testis and ovary of *P. sophore*. Primers for the *hsps* 90, 70, and 60 were adapted from Mahanty et al. 2016b. Primers for *oct-4* and *sox-2* were adapted from Wagner and Podrabsky (2015). Primers for *nanog* and *vtg* were adapted from Zhendong (2009) and Henry et al. (2009), respectively. Lyophilized primers were procured from Integrated DNA Technologies (USA) and were reconstituted using nuclease-free water. Information of the sequences of the primers, annealing temperature, and accession numbers are listed in Table 1. The specificity of the primer sets was confirmed by the presence of a single band of appropriate size obtained after PCR amplification.

qPCR analysis

The real-time PCR amplifications were carried out using SYBR Green detection chemistry. cDNA were run in triplicates on a 96-well reaction plates with the CFX Connect real-time PCR (Bio-Rad, UK). Twenty microliter of reaction mixture contained 10 μl of VeriQuest SYBR Green Mix (Bio-Rad, UK), 1.0 μl of each 10 μM of primers and 5 μl of diluted cDNA as template and 3 μl of RNase/DNase-free sterile water (Thermo Scientific, USA).

The following amplification programs were used in all RT-qPCR reactions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 15 s, and 95 °C annealing and extension for 45 s at optimized temperatures for specific candidate genes. The specificity of each amplification reaction was verified by a melting curve analysis after 40 cycles. No template controls (NTC) were included for each primer pair for cross-checking any possible contamination of assay reagents.

PCR efficiency of the genes was determined by a standard curve analysis of cDNA samples according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline (Bustin et al. 2009). A series of 10-fold dilution of three replicates of cDNA was made to determine the gene-specific PCR amplification efficiency for each primer pair used in qPCR experiments. Standard curve was derived from the E values by the formula $E = 10^{-1/\text{slope}}$. The mean efficiency values were obtained for each tissue samples and were used to adjust the quantitative cycle (Ct) values for further analysis.

Expression analysis of ten candidate reference genes was earlier carried out, and *eef1* and *b2mg* were found to be the most suitable reference genes in ovary and testis, respectively (Mahanty et al. 2017). Thus, these two genes were used for normalization of expression of the target genes (*hsps*, *sox-2*, *oct-4*, *nanog*, *vtg*, *dmrt-1*) in the respective tissues. The comparative Cq (delta Cq) method was used to calculate the changes in gene expression as a relative-fold difference between the control and treated sample. MIQE guidelines were followed for the qPCR experiments (Bustin et al. 2009).

Statistical analysis

Fold changes of gene expression are expressed in comparison with the control. One way analysis of Variance (ANOVA) followed by Tukey's test was employed to compare the variation between the experimental groups ($p < 0.05$).

Table 1 Primer sets used for RT-qPCR analysis

Gene	Primer sequence (5′–3′)	Annealing temperature	Accession no.	Reference
hsp90	F: 5′-GGAAATCTTCCTCCGAGAGC-3′ R: 5′-CCGAATTGACCGATCATAGA-3′	51 °C	KC962223	Mahanty et al. 2016b
hsp70	F: 5′-GCATGGTGAACCACTTTGTG-3′ R: 5′-CTCTGCCGTTGAAGAAATCC-3′	53 °C	JX401427	Mahanty et al. 2016b
hsp60	F: 5′-C(C/T)GTCACCATGGG(A/G/T)CCAAAGG-3′ R: 5′-C/G/T)GCCTCTCCATCCACATCC/T)TC(A/C)GC-3′	65 °C	KC844065	Mahanty et al. 2016b
vtg	F: GGTGACTGGAAGATCCAAG R: TCATGCGGCATTGGCTGG	55 °C	KU533862	Henry et al. 2009
sox-2	F: CAAGACCCTCATGAAGAAGGAC R: TBCAGTACAACTCCATGACYA	50 °C	EF431920.1	Wagner and Podrabsky 2015
oct-4	F:GAGGCTCTSCARCTKAGYTTC R:RGATGTRGTVCGWGTDTGGTT	53 °C	NM_131112.1	Wagner and Podrabsky 2015
nanog	F: GAGCGCTTCAATCAGCATCC R: GTTAAGTTCCGTTCTCCACTGTC	55 °C	EF550998	Zhendong 2009
dmrt1	F: ATGGTGGACGCCTCCTATTAC R: AGGGCAGGTGCTGGGTTG	60 °C	AY157562.1	Zhang et al. 2013

Results

Expression of *hsp* genes in gonadal tissues

In ovary, the expression of *hsp70* and *hsf1* was found to be downregulated in both the heat-stressed groups (both short-term and long-term). *hsp60* was found to be significantly upregulated, whereas the expression of *hsp90* was found to be downregulated in the 7-day heat-exposed fishes. Expressions of both *hsp60* and *hsp90* were found to be returning to the basal level in the fish exposed to heat stress for 60 days.

Similar to ovary, in testis also, the expressions of *hsp70* and *hsf1* were found to be downregulated in both the heat-stressed groups. But unlike the ovarian tissues, in testis, expressions of *hsp60* and *hsp90* were found to be downregulated in both the heat-stressed groups and no signs of recovery were found in the 60-day heat-exposed groups (Fig. 1).

Expression of pleuripotency marker genes (*sox-2*, *nanog*, *oct-4*)

In both ovary and testis, significant downregulation in expression of *sox-2*, *nanog*, and *oct-4* was observed in the heat-stressed groups. There was a gradual decrease in expression of these genes with increase in time period of heat exposure (Fig. 2).

Expression of *vtg* and *dmrt-1*

Expression of *vtg* remained unaltered in both ovary and testis of fishes heat stressed for 7 days while significant downregulation was observed in gonadal tissues of fishes exposed to heat stressed for 60 days. In ovary, the expression of *dmrt-1* (double sex mab-3–related transcription factor 1) was found to be downregulated in the heat-stressed groups, while in testis, the expression of *dmrt-1* was upregulated in the fishes heat stressed for 7 days but found to be returning to the basal level in the 60 days exposed fishes (Fig. 3).

Discussion

Expression of *hsps*

Hsps play important roles in maintaining physiological integrity by stabilizing, refolding the denaturing proteins, and facilitating the proteolysis of the denatured proteins (Sottile and Nadin 2017; Wang et al. 2015; Zunino et al. 2016; Mahanty et al. 2016a, b). The *hsps* are known to be expressed in various cell types including gonadal cells, and studies suggest that some of the *hsps* play fundamentally important roles during early development. Hsps are known to be expressed in normal cells, but their expression is increased during stressed condition. However, in case of gonadal (ovary) tissues, a number of studies have shown the decrease in

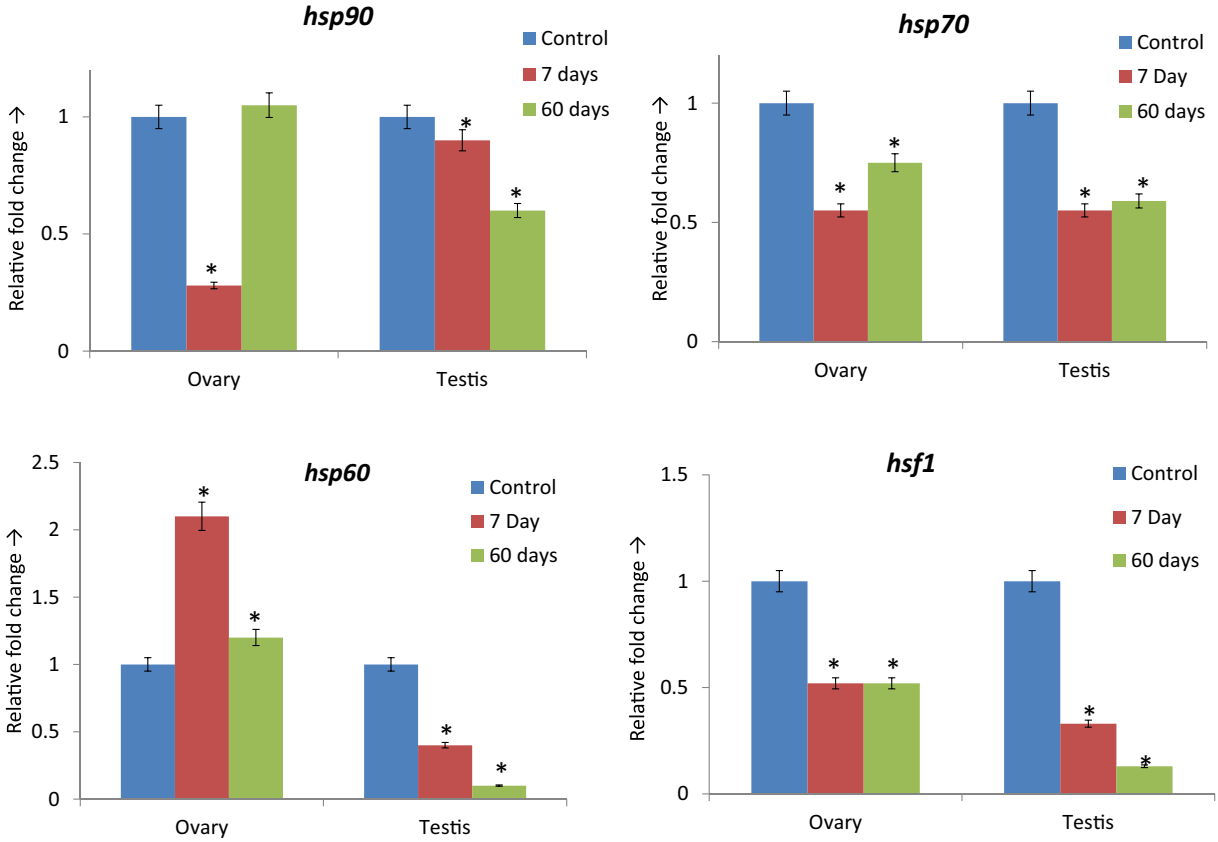

Fig. 1 Expression profile of *hsp* genes in gonadal tissues of *Puntius sophore* heat stressed for 7 and 60 days. The *hsps* were found to be downregulated in the gonadal tissues of heat-stressed fish except *hsp60* in ovary. (*) above the bars indicate significant difference in the values in comparison to control ($p < 0.05$)

abundance of the *hsps* as a marker of poor egg quality (Kohn et al. 2015; Chapman et al. 2014). Kohn et al. (2015) have reported a decrease in abundance of *hsp70* in poor-quality eggs in *Polyprion oxygeneios*. Chapman et al. (2014) identified *hsp90* transcripts among a subset of 233 mRNA species whose abundance had predictive value for egg quality in striped bass. "Egg quality" has been defined as the ability of the egg to be fertilized and subsequently develop into a normal embryo (Bobe and Labbe 2010), and poor-quality eggs are those which have very less ability to get fertilized and subsequently develop into a viable embryo (Bobe 2015). In the present study also, we found that downregulation in the expression of *hsp70*, and *hsf1* could be indicative of deteriorative changes in the ovarian functionalities that could lead to production of poor quality eggs. Along

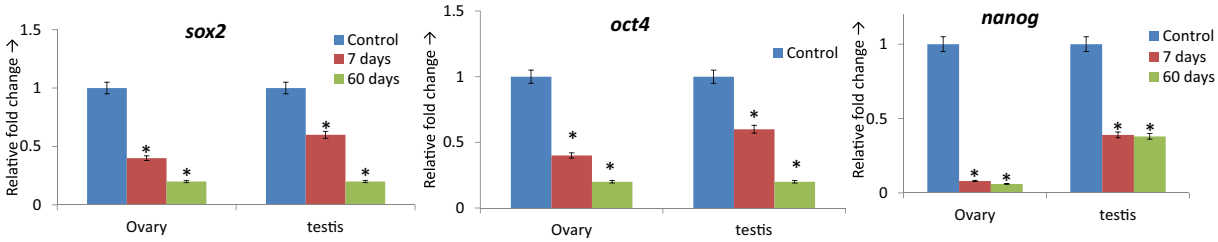

Fig. 2 Expression profile of pleuripotency marker genes *sox2*, *oct4*, and *nanog* in gonadal tissues of *Puntius sophore* heat stressed for 7 and 60 days. Downregulation in expression of the pleuripotency marker genes was observed in the gonads of heat-stressed fishes indicating loss of pleuripotency of gonadal cells. (*) above the bars indicate significant difference in the values in comparison to control ($p < 0.05$)

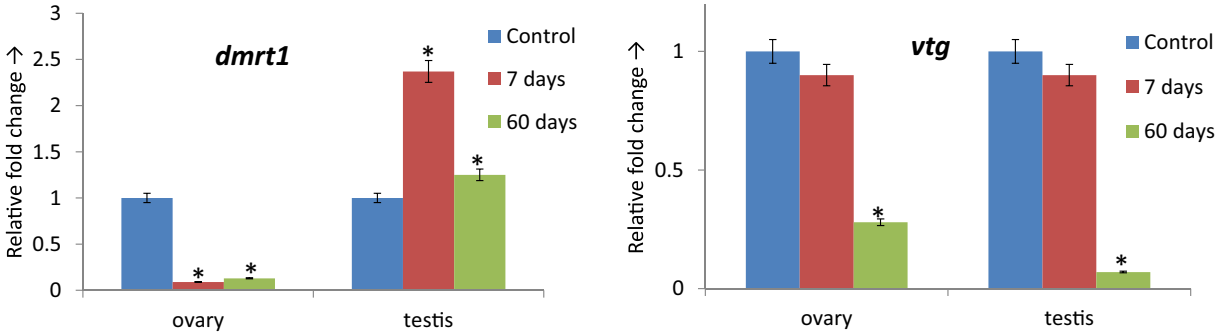

Fig. 3 Expression profile of sex determining gene dmrt1 and gene involved in vitellogenesis *vtg* in gonadal tissues of heat-stressed *Puntius sophore*. *dmrt1* was found to be downregulated in ovary and upregulated in the testis of heat-stressed *Puntius sophore* indicating that high temperature might favor production of higher number of males. (*) above the bars indicate significant difference in the values in comparison to control ($p < 0.05$)

with that, significant alterations in expression of *hsp90* and *hsp60* were observed in ovary of fishes exposed to heat stress for 7 days (down- and upregulations, respectively) which were found to be returning to basal level after 60 days of heat exposure. This could be an indication of acclimatization to warm temperature after a relatively longer period of time.

In testes, all the *hsps* were found to be downregulated in the heat-stressed groups. Studies have shown that downregulation of *hsps* in testes lead to apoptosis of testicular cells (He et al. 2010). The downregulation in expression of *hsps* could be because of the progression of testicular cells to apoptotic pathways. Similar to the present findings, Domingos et al. (2013) have shown downregulation of *hsps* in testes of fishes during warmer seasons of year.

Expression of pleuripotency marker genes

The *sox-2*, *oct-4*, and *nanog* are transcription factors that regulate transcription of proteins of the leukemia inhibitory factor (LIF) signaling pathway (Magnani and Cabot 2009). These three transcription factors contribute to a complex molecular network necessary for maintenance of cellular pluripotency. Pleuripotent stem cells are necessary for the maintenance of many adult tissues including the gonads (Greenspan et al. 2015). Gonads are the only organs capable of transmitting genetic materials to the offspring and are composed of both somatic and germline stem cells (Liu et al. 2009). However, pleuripotency of the gonadal stem cells and their self-renewal capability largely depend on their microenvironment and any misregulation in the signaling pathways in this microenvironment can lead to depletion of different stem cell pools (Greenspan et al. 2015). In this context, we analyzed the expression profile of three pleuripotent stem cell marker genes *sox-2*, *oct-4*, and *nanog* in both testis and ovary of fish exposed to heat stress. All these genes were found to be downregulated in both ovary and testis of *P. sophore* exposed to heat stress which indicates depletion in the number of stem cells; the stem cell could possibly be either undergoing apoptosis, or these cells might be losing their pleuripotency.

Expression of *vtg* and *dmrt-1*

Vtg gene synthesizes vitellogenin which is the precursor protein of yolk. Vitellogenin is a protein that is generally synthesized in female fishes; however, in presence of endocrine disruptive chemicals (EDC), it can be synthesized in male fish also (Hara et al. 2016). Vitellogenin has been found to be a marker of egg quality, and its downregulation in ovary is indicative of poor egg quality (Kohn et al. 2015) and one of the markers used for presence of endocrine disruptive chemicals (Endocrine disruptor screening and testing advisory Committee 1998; Marin and Matozzo 2004; Klaper et al. 2006). In the present study, we observed downregulation in expression of *vtg* in ovary of fish exposed to heat stress but its expression was inconsistent in testis. The downregulation of *vtg* in ovary indicates that, like EDCs, high temperature stress could also disrupt the endocrine functions and can hamper the egg production process. This inference has been made on the basis of the downregulation of *vtg* in the gonads of heat-stressed fish. However, the possibility that high temperature acts as an endocrine disruptor merits further investigation.

Sex in fish is plastic and in several species can be influenced by environmental factors (Díaz and Piferrer 2015). Thus, we studied the expression of *dmrt-1* in both the gonads of fish exposed to high temperature stress to know whether high temperature supports masculinization or feminization. In case of zebrafish, which is also a cyprinid like *Puntius*, *dmrt-1* plays an important role in male sex determination and testis development (Webster et al. 2017; Lambeth et al. 2014). Webster et al. have shown that the mutations in *dmrt-1* gene result in complete sex reversal from male to female in zebrafish. It not only plays a role in genotypic sex determination but also plays important functions in environmental sex determination especially in temperature-dependent sex determination (Fernandino et al. 2008). The expression of *dmrt1* was upregulated in testes and downregulated in ovary of the heat-stressed fishes which could be possibly because of a masculinization supportive role of high temperature. In many fish species, temperature-dependent sex determination (TSD) has been observed along with genetic sex determination (GSD) which varies among the species (Baroiller et al. 2009). In some species like tilapia, high temperature above 32 °C favors feminization (Baroiller et al. 2009), whereas in species like *Danio rerio*, *Oryzias latipes*, and *Onchorhynchus nerka*, high temperature favors masculinization. The present study suggests that high temperature could favor masculinization in *P. sophore* like its close relative *D. rerio* (Ospina-A lvarez and Piferrer 2008). However, fish in general have a weak genetic component of sex determination and the mechanisms of sexual differentiation may vary among fish species. Thus, this merits further investigation to see if *dmrt-1* is involved in sex determination in *Puntius* also and if the upregulation in its expression has any male favoring effect.

The present study showed that even though expression of some of the genes had recovering tendency with prolonged period of temperature stress, most of the genes had altered expression indicating a negative impact on the reproductive physiology of the fish. As observed in the present study, anomalous temperature has been found to be having an inhibitory effect on reproductive physiology of the fish *Takifugu niphobles* through suppression in expression of a number of genes like *kisspeptin and gnrh* (Shahjahan et al. 2017). Similarly, Díaz and Piferrer (2017) have reported that temperature has a masculinizing effect which is overridden by estrogen exposure.

Conclusion

The present study showed that with increasing temperature, there was a downregulation of *hsps* indicating that high temperature can affect the quality of gametes produced. Similarly, the downregulation of pleuripotency marker genes in the heat-stressed fish suggests that the gonads could lose their pleuripotent cells thereby affecting the gonadal integrity. This study suggests that the reproductive physiology and health of the nutrient dense *P. sophore* could be negatively affected with rise in environmental temperature.

Acknowledgements A.M. and G.K.P. were NFBSFARA Senior Research Fellows. The authors are thankful to the Director, ICAR - Central Inland Fisheries Research Institute, Barrackpore, and the Director, School of Biotechnology, KIIT University, Bhubaneswar, for the facilities and encouragement. Technical assistance received from Mr. Laddu Ram Mahaver, Mr. Rabiul Sk. Mr. Ravi Sonkar, and Mr. Sujoy Kumar Nath is acknowledged. The authors acknowledge the valuable comments received from the reviewers which contributed immensely to the improvement of the manuscript.

Funding information This research was funded by the Indian Council of Agricultural Research under the National Fund for Basic, Strategic and Frontier Application Research in Agriculture (NFBSFARA; recently renamed National Agricultural Science Fund, NASF) Project No. AS-2001 (B.P.M. and S.M).

Compliance with ethical standards The study including sample collection, experimentation, and sacrifice met the ethical guidelines including adherence to the legal requirements of the study country. The study was approved by the Institute Animal Ethics Committee (IAEC) of Central Inland Fisheries Research Institute vide approval no. CIFRI/IAEC-17/03.

Conflict of interest The authors declare that they have no conflicts of interests.

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