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Physiological stress biomarkers reveal stocking density effects in late larval Delta Smelt (*Hypomesus transpacificus*)

Matthias Hasenbein [a,b,c], Nann A. Fangue [b], Juergen P. Geist [c], Lisa M. Komoroske [a,b], Richard E. Connon [a,*]

[a] Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA 95616, USA
[b] Department of Wildlife, Fish & Conservation Biology, University of California, One Shields Avenue, Davis, CA 95616, USA
[c] Aquatic Systems Biology Unit, Department of Ecology and Ecosystem Management, Technische Universität München, Mühlenweg 22, D-85354 Freising, Germany

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ABSTRACT

Suboptimal fish stocking densities in experimental systems may elicit stress responses that can affect experimental results. Fish species, age and size, water chemistry and flow, and physical characteristics of the experimental system (e.g., tank, cage) are among the parameters to be considered when determining stocking densities. However, systematic studies to define fish densities minimizing stress in experimental systems are rarely performed. This is particularly true when working with species of low aquaculture value or a non-model test species such as the Delta Smelt (*Hypomesus transpacificus*). The aim of this study was to use physiological stress biomarkers to determine suitable fish densities for specific experimental vessels routinely used for this species. We maintained late larval Delta Smelt (60 days post-hatch; dph) over a period of 24 h, at five different densities: 7, 14, 28, 42, and 56 fish per 8 L circular fish tank. We assessed whole body cortisol and transcriptomic biomarkers that lead to cortisol production to quantify stress levels. Both marker types delivered similar results. Cortisol levels were lowest at densities of 28 and 42 fish per tank, whereas lowest fish densities (7 and 14 fish per tank) evoked the highest stress levels. Genes such as Mineralocorticoid Receptor 1 and Glucocorticoid Receptor 2, as well as 11-Beta-Hydroxysteroid-Dehydrogenase-2 depicted the lowest expression levels at stocking densities 28 and 42, and elevated expression levels for stocking densities 7 and 14. Our data support the observations that late larval Delta Smelt should be exposed, acclimated, and cultured in groups rather than as individuals or in low numbers. This study indicates the importance of adequately defining experimental conditions that minimize stress, specifically when stress is measured as an endpoint. In addition to classical cortisol measurements, responses of the transcriptome also appear suitable in assessing stress responses in fish, and in determining optimal holding conditions, particularly if short-term responses are the study focus.

Statement of relevance

The study highlights the importance of evaluating stress in order to determine species-specific stocking densities. The results are thus relevant to a wide audience in the field of aquaculture and experimental biology.

1. Introduction

Fish stocking density is known to affect physiological and behavioral responses in fishes, and previous studies have utilized a variety of endpoints to measure these responses (Adams et al., 2007; Ashley, 2007; Ellis et al., 2002; Martins et al., 2012; Turnbull et al., 2005). Commonly applied methods used in density studies include assessments of parameters involved in aquaculture production and nutritional status (mortality, body condition factor, food intake, food conversion rate, growth, and size variation), health condition (blood parameters, fin damage, gill condition, and spleen condition), as well as stress indicators (cortisol and glucose concentrations, and elevated oxygen consumption) (Ellis et al., 2002; North et al., 2006). Some endpoints, such as those involved in aquaculture production, nutritional status, health condition, and behavior can take longer to manifest and are thus predominantly applied to experiments lasting several weeks to months (Adams et al., 2007; Aketch et al., 2014; Alanärä and Brännäs, 1996; Boujard et al., 2002; Brown et al., 1992; de Oliveira et al., 2012). In contrast, other parameters involved in the immediate stress response, e.g., cortisol levels and gene expression, change rapidly and can be used to determine short term stress and stress recovery at time points ranging from several hours to a few days (Caipang et al., 2008a, b; Fast et al., 2008). The use of molecular biomarkers as stress indicators in aquatic organisms has been successfully demonstrated in various studies addressing stress resulting from turbidity and salinity (Hasenbein et al., 2013), contaminants (Connon et al., 2011a; Garcia-Reyero et al., 2008; Geist et al., 2007), density effects (Caipang

* Corresponding author at: University of California, Davis, School of Veterinary Medicine, APC, 1089 Veterinary Medicine Drive, Davis, CA 95616, USA.
E-mail addresses: mhasenbein@ucdavis.edu (M. Hasenbein), reconnon@ucdavis.edu (R.E. Connon).

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et al., 2008a, b; Gornati et al., 2004a, b; Salas-Leiton et al., 2010), as well as recovery from stress (Wiseman et al., 2007). Besides commonly assessed plasma cortisol levels (e.g., Pickering and Pottinger, 1989), whole-body cortisol levels have also been used successfully to determine stress levels in fish (Cachat et al., 2010; Ramsay et al., 2006, 2009), but few studies have associated them with responses on the transcriptome.

Since stocking density impacts physiological and behavioral responses in fishes, defining suitable stocking densities is important in aquaculture, as well as in experimental investigations testing biologically-relevant hypotheses. In addition, studies on several different fish species using the same experimental protocol revealed species-specific differences in the intensity and direction of the stress response after density manipulation (Pottinger, 2010). Stress and aggression can increase at low densities when social hierarchies instead of schooling behaviors occur. On the other hand, high stocking densities can also result in an increase in stress levels due to intraspecific competition. Studies on different species and different experimental settings have indicated that both high and low stocking densities can cause severe stress in fish (Aketch et al., 2014; Alanärä and Brännäs, 1996; Brown et al., 1992; Costas et al., 2008; Jørgensen et al., 1993).

Generalized stress responses in fishes can be activated by diverse and sometimes subtle stressors (reviewed in Barton, 2002; Wendelaar Bonga, 1997), and any additional stress, such as one resulting from unfavorable stocking density, can act as a confounding factor impacting experimental results, which in turn can potentially lead to misinterpretation of data. Thus assessing density stress is particularly crucial when evaluating stress responsive parameters (e.g., cortisol, glucose) as experimental endpoints. Moreover, any experimental protocol should also try to minimize stress of the organisms for animal welfare reasons. Experimental stocking densities, however, are often exclusively based on numbers of individuals needed for specific assessments; e.g., pooling organisms to achieve a certain yield (Alsop and Vijayan, 2008), accepted sample sizes (n-values) (Jensen, 1972), or empirical observations (e.g., replication, independence of samples to increase statistical power). For instance, in ecotoxicological testing, the number of organisms used per treatment tends to follow specific guidelines such as standard protocols implemented by the U.S. Environment Protection Agency (USEPA, 2002), the Organization for Economic Cooperation and Development (OECD, 2006), or the International Organization of Standardization (ISO, 2014). Furthermore, there is a requirement that researchers seek reasonable alternatives concerning the use of animals in experimentation, failing which there is an obligation to minimize numbers used (Schechtman, 2002).

Experimental protocols do not always account for the physiological impacts of stocking density. For example, Oikari (2006) reviewed the use of caging techniques in field-based ecotoxicological studies, and highlighted that stress susceptibility in fish is rarely incorporated into toxicological evaluations. The review further indicates that test acceptance standards are usually based on survival, regardless of density, thus somewhat biased results are likely accepted. Oikari (2006) further recommends the development of suitable and stress-free stocking densities, in particular when assessing physiological responses associated with organismal stress as an endpoint to answer a specific research question.

A wealth of information on stocking density effects is available for aquaculture-relevant species, from which experimental stocking densities that minimize stress can be derived. Examples of such species include salmon (*Salmo salar*) (Adams et al., 2007; Turnbull et al., 1998, 2005), rainbow trout (*Oncorhynchus mykiss*) (Ellis et al., 2002), Arctic charr (*Salvelinus alpinus*) (Brown et al., 1992; Jørgensen et al., 1993) or model species such as zebrafish (*Danio rerio*) (Gronquist and Berges, 2013; Pavlidis et al., 2013; Spence and Smith, 2005), fathead minnow (*Pimephales promelas*) (Ankley and Villeneuve, 2006; Smith et al., 1978), and Japanese medaka (*Oryzias latipes*) (Davis et al., 2002; USEPA, 1991). When working experimentally with non-model and/or endangered fish species, data on optimal stocking densities may be limited, and thus important to determine. Here, we use the Delta Smelt (*Hypomesus transpacificus*) as an example non-model fish to demonstrate the importance of stocking density determination in experimental biology.

The Delta Smelt is a fish species endemic to the Sacramento–San Joaquin River Delta, California, USA, and was listed as threatened in 1993 under both State and the Federal Endangered Species Acts (CDFW, 2014; USFWS, 1993) and as endangered under California State Endangered Species Acts in 2010 (CDFW, 2014). In response to the species' decline, the University of California, Davis Fish Conservation & Culture Laboratory (FCCL) was founded with the mission to develop intensive fish culture techniques to create a genetically and demographically robust captive population and provide a supply of fish for research purposes (Fisch et al., 2013; Lindberg et al., 2013). Information on appropriate stocking densities for this species beyond those used for facility production (Baskerville-Bridges et al., 2005; Lindberg et al., 2013) is still limited, yet there is an increasing number of studies that use this species in ecotoxicological testing and ecophysiological testing (Connon et al., 2009, 2011a, b; Hasenbein et al., 2013; Komoroske et al., 2014; Swanson and Young, 1998; Swanson et al., 2000). We have used this species towards establishing links between density-dependent physiological stress responses, with the aim of estimating the stocking densities that minimized stress in late larval Delta Smelt for a commonly used experimental design. Because Delta Smelt are described as an aggregating pelagic species (Bennett, 2005; Moyle, 2002), we hypothesize that the stress response will be elevated at low densities, as well as at high stocking densities. Therefore, this study aims to determine suitable densities to be used for experimental designs.

2. Material & Methods

2.1. Fish exposures

Fish and experimental space were provided by the University of California, Davis Fish Conservation and Cultural Laboratory (FCCL; Byron, CA, USA). The fish were reared according to the methods described in Lindberg et al. (2013). In brief, fish were cultured in black tanks with no substrate, plants, or structure. The larvae were maintained in a recirculating system with biofilters, and fed with *Artemia franciscana*, 6 times per day at a volume of 1–3 nauplii/mL. Fish were held at densities between 4 and 8·L^{-1} at an age of 40–50 dph. At early larval stages fish were stocked at 42–46 fish ·L^{-1}. The light intensity is kept low to accommodate the light sensitivity and ranges depending on life stage between 10 and 50 lx. The average rearing temperature across the rearing period was 17.4 °C (SD ± 0.66). The mean length (fork length) and weight of 60 dph Delta Smelt were 19.37 mm (SD ± 2.69) and 0.0303 ·*g* (SD ± 0.015). Late larval Delta Smelt (60 dph) were exposed in triplicate, in aerated facility water at densities of 7, 14, 28, 42, or 56 fish per black circular fish tank (8 L) (2 gal black plastic pail; item # 3539; United States Plastic Corporation®, USA; Dimensions: height: 23.90 cm, top outer diameter: 24.20 cm, bottom outer diameter: 23.90 cm) for a period of 24 h at a light:dark cycle of 16 h:8 h. Tanks were aerated with air stones during the entire experiment. Tanks were static and water level in tanks was adjusted to a height of 19.50 cm which results in a total water volume of 6.4 L per tank. Fish were fed prior to the experiment, but were kept unfed throughout the test duration in order to avoid stress variability related to feeding. All handling and experimental procedures were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC protocol # 16591). Dissolved oxygen, salinity, specific conductance, pH, turbidity, ammonia (total ammonia-N), and light intensity were monitored at test initiation and at test termination (Table 1). Temperature was recorded throughout the experiment using iBCod submersible temperature loggers (Alpha Mach Inc., Ste-Julie, QC, Canada). Tank control fish were sampled using a 1 L beaker directly

from the facility rearing tank previous to test initiation to determine baseline stress, following the same process used to transfer fish to the experimental tanks. At experiment termination, fish were immediately euthanized with an overdose of 50 mg L^{-1} tricaine methanesulfonate (MS-222; Finquel, Argent Laboratories, Redmond, WA, USA), at neutral pH, buffered with sodium bicarbonate. Whole fish were transferred into 1.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Samples were stored at −80 °C for subsequent biochemical and molecular analyses.

2.2. Cortisol assessments

Whole body cortisol was assessed in a total of nine fish from each treatment (three per replicate), using the methods established for zebrafish (Alsop and Vijayan, 2008; Cachat et al., 2010); volumes of solutions used were optimized for use in Delta Smelt (Hasenbein et al., 2013). In brief, samples were defrosted on ice and homogenized for 2 min in 1 mL ice-cold 1× PBS buffer (phosphate buffered saline, BioUltra, Sigma Aldrich®, lot # BCBD7118) using a TissueLyser LT (Qiagen©, Venlo, Limburg, Netherlands) at a frequency of 50 Hz. The resulting homogenate was divided in equal amounts of 500 μL, and used for cortisol and total protein determinations. Samples were processed at 4 °C throughout the complete extraction procedure. Cortisol was extracted from the homogenate by adding 2.5 mL diethyl ether (VWR International LLC, Radnor, PA, USA) and subsequent vortexing for 1 min. A total of three washing steps with diethyl ether were performed in order to achieve the maximum yield. After each washing step, samples were centrifuged immediately for 7 min at 3200 ·*g* at 4 °C using a refrigerated centrifuge (Sorvall Biofuge Primo R, Kendro Laboratory Products, Hanau, Germany). The supernatant was transferred to a 15 mL Pyrex glass tube for diethyl ether evaporation, which was performed using an air pump with a 0.22 μm Millipore Express filter (EMD Millipore Corporation, Billerica, MA, USA). Dried samples were resuspended in 200 μL ice-cold 1× PBS buffer and incubated overnight at 4 °C for resuspension. Cortisol assays (Salivary Cortisol, Enzyme Immunoassay Kit, Salimetrics, Inc., State College, PA, USA) were performed according to the manufacturer's instructions, and cortisol levels (mg · dL^{-1}) were calculated with a four-parameter sigmoid standard curve (minus curve fit). The cortisol assay has been validated for Delta Smelt (Hasenbein et al., 2013) and zebrafish (Cachat et al., 2010; Egan et al., 2009), with a cross reactivity of the antibody for cortisone of 0.13%. Cortisol levels were normalized to total protein and denoted as cortisol concentration (pg cortisol ·μg $protein^{-1}$). The second half of the homogenate was used to determine protein content. Following centrifugation at 16,500 ×*g* for 30 min at 4 °C (Beckman Allegra 21R Centrifuge, Beckman Coulter Inc., Indianapolis, IN, USA) the supernatant of each sample was collected and used for total protein content determination following the manufacturer's protocol (BCA Protein Assay Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Quantitative PCR

Total RNA was extracted from whole-body homogenates. RNA extractions were performed according to the manufacturer's protocols using the RNeasy Mini QIAcube Kit (Qiagen©, Venlo, Limburg, Netherlands) utilizing a QIAcube (Qiagen©, Venlo, Limburg, Netherlands). Qualitative and quantitative RNA determinations were conducted using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA); 260/280 and 260/230 ratios ranged from 1.98 to 2.20, and from 1.45 to 2.35, respectively. Integrity of total RNA was assessed by electrophoresis on a 1% (w/v) agarose gel stained with SYBR Dye (SYBR Safe DNA Gel Stain, Invitrogen™, Life Technologies™, Carlsbad, CA, USA). Eight to nine fish per treatment (2–3 from each replicate dependent on survival) were assessed by quantitative polymerase chain reaction (qPCR). Complementary DNA (cDNA) synthesis was performed with 1 μg total RNA per sample, using Reverse Transcriptase III (SuperScript® III Reverse Transcriptase, Invitrogen™, Life Technologies™, Carlsbad, CA, USA). Primer and probes for qPCR analyses were designed using Roche Universal Library Assay Design Center (https://www.roche-applied-science.com). Designed primers were purchased through Eurofins MWG Operon (http://www.eurofinsdna.com), and fluorescent

Table 1
Physicochemical water parameters. Means with standard deviations across all densities and replicates over the 24 h exposure time.

Parameter, unit and time point	Mean/SD ±	Facility water	Density 7	Density 14	Density 28	Density 42	Density 56
DO mg ·L^{-1}/T0	Mean	9.8	9.5	9.4	9.4	9.2	9.1
	SD	0.3	0.0	0.0	0.0	0.1	0.1
DO mg ·L^{-1}/T24	Mean	N/A	10.1	10.0	10.0	9.9	9.9
	SD	N/A	0.1	0.1	0.1	0.1	0.1
SC μS ·L^{-1}/T0	Mean	497	494	499	496	493	496
	SD	4	1	1	4	2	2
SC μS ·L^{-1}/T24	Mean	N/A	500	501	499	498	502
	SD	N/A	3	3	3	2	2
pH/T0	Mean	7.9	8.0	8.0	8.0	8.0	8.0
	SD	0.2	0.0	0.0	0.0	0.1	0.3
pH/T24	Mean	N/A	7.7	7.7	7.8	7.8	8.0
	SD	N/A	0.0	0.0	0.1	0.1	0.2
Sal PSU/T0	Mean	0.2	0.2	0.2	0.2	0.2	0.2
	SD	0.0	0.0	0.0	0.0	0.0	0.0
Sal PSU/T24	Mean	N/A	0.2	0.2	0.2	0.2	0.2
	SD	N/A	0.0	0.0	0.0	0.0	0.0
Tur NTU/T0	Mean	13.1	13.7	14.0	13.6	13.5	14.0
	SD	0.7	0.1	0.4	0.9	0.4	0.5
Tur NTU/T24	Mean	N/A	12.5	13.5	12.9	13.0	13.4
	SD	N/A	0.5	0.2	0.6	0.3	0.6
TAN mg ·L^{-1}/T0	Mean	0.1	0.1	0.1	0.1	0.1	0.1
	SD	0.0	0.0	0.0	0.0	0.0	0.0
TAN mg ·L^{-1}/T24	Mean	N/A	0.2	0.2	0.2	0.2	0.3
	SD	N/A					
T °C	Mean	16.6	15.8	15.8	15.8	15.8	15.8
	SD	0.2	0.7	0.7	0.7	0.7	0.7

Abbreviations: DO (dissolved oxygen, mg·L^{-1}), SC (specific conductance, μS·cm^{-1} adjusted to 25 °C), pH-value, Sal (salinity, practical salinity unit; PSU), Tur (turbidity, nephelometric turbidity units; NTU), TAN (total ammonia-N), T (temperature, °C), SD ± (standard deviation). N/A indicates that values are not applicable. T0 = time point at test start, T24 = time point at test termination.

Table 2
Primer and probe sequences of genes used as molecular biomarkers to determine stress levels in late larval Delta Smelt (*Hypomesus transpacificus*).

Gene name	Gene code	Primer 5′ → 3′	Primer 3′ → 5′	Probe #	% efficiency
Glutathione-S-Transferase	GST	aatctccctggcagacattgtt	ggccggctctcaaacacat	127	108
Mineralocorticoid Receptor 1	MR1	tttctacactttccgcgagtca	tgatgatctccaccagcatctc	39	99
Glucocorticoid Receptor 2	GR2	catcgtgaagcgtgaggagaa	tgcatggagtccagtagtttgg	129	98
Pro-opiomelanocortin	POMC	tgttcacctgtgcaggtctga	gagaagctctcttccgtggaca	127	102
11-Beta-Hydroxysteroid-Dehydrogenase Type 1	11-Beta-HSD-1	cgtgtcgtctctgctggcta	ggcgaacttggtggaggag	55	109
11-Beta-Hydroxysteroid-Dehydrogenase Type 2	11-Beta-HSD-2	tcctgccatcctcctacaagac	tctggaccaggtgtttgaactg	14	106
Beta Actin	β-Actin	tgccacaggactccatacc	catcggcaacgagaggtt	11	107
Glyceraldehyde-3-phosphate Dehydrogenase	GAPDH	tccacgagaaagacccaact	cacgccagtagactcaacca	159	95

probes were obtained from Applied Biosystems (Applied Biosystems®, Life Technologies™, Carlsbad, CA, USA). Quantitation of transcription was performed using SDS 2.4 software (Applied Biosystems®, Life Technologies™, Carlsbad, CA, USA). Responding genes were normalized using a normalization factor calculated based on the geometric mean of two control genes; *Glyceraldehyde-3-phosphate Dehydrogenase* (GAPDH) and *elongation-factor alpha* (EF-α). Normalization was performed according to the "geNorm" algorithm version 3.5 as described in Vandesompele et al. (2002). Genes were selected based on their involvement in the HPI-axis (Table 2). *Pro-opiomelanocortin* (POMC) is the precursor of adrenocorticotropic hormone (ACTH) that binds to melanocortin receptor 2 (MC2R) in interrenal cells, which in turn stimulates cortisol production through adenylate cyclase and cAMP-dependent signaling pathways (Alsop and Aluru, 2011). Cortisol signaling is further mediated by two important receptors, *Glucocorticoid Receptor 2* (GR2) and *Mineralocorticoid Receptor 1* (MR1), which are both ligand-activated transcription factors (Alsop and Vijayan, 2008; Prunet et al., 2006). Mineralocorticoids and glucocorticoids bind to MR1 and GR2 inducing the stimulation or repression in transcription of target genes (Tomlinson et al., 2004). *11-Beta-Hydroxysteroid-Dehydrogenase-Type 1* (11-Beta-HSD-1) and *11-Beta-Hydroxysteroid-Dehydrogenase-Type 2* (11-Beta-HSD-2) are the genes coding for two enzymes which are directly involved in the activation and inactivation of glucocorticoids; e.g., cortisol (Krozowski et al., 1999). In particular, 11-Beta-HSD-1 is a bidirectional enzyme that functions first and foremost as a reductase by converting cortisone into cortisol and also can function as a dehydrogenase by converting cortisol into cortisone (Krozowski et al., 1999; Tomlinson and Stewart, 2001). In contrast, 11-Beta-HSD-2 is a unidirectional enzyme that converts cortisol into cortisone (Krozowski et al., 1999; Tomlinson and Stewart, 2001), and protects MR1 from occupation by cortisol (Stewart and Mason, 1995; Tomlinson and Stewart, 2001). In addition, *Glutathione-S-Transferase* (GST) a key enzyme for biotransformation (Choi et al., 2008), was measured. GST defends cells against reactive oxygen species (ROS) and responds to osmotic stress in fishes (Choi et al., 2008).

2.4. Statistical analysis

Data analysis was performed using R version 3.0.2 for statistical computing (R-CoreTeam, 2014) and associated packages. Cortisol data and gene expression data were tested for normal distribution using the Shapiro Wilk normality test and homogeneity of variances was determined using the Fligner–Killeen Test. Cortisol data were log-transformed to meet assumptions of normality. Effects of density on cortisol levels and transcription of individual genes were evaluated with nested analysis of variance (ANOVA) tests using lme4 (Bates et al., 2014) as described in Ruohonen (1998). Density was defined as a fixed categorical main effect, and experimental replicate (bucket) was treated as a random effect nested within each density treatment. Statistical significance was determined using a significance level of $\alpha = 0.05$ for all tests. Post hoc contrasts were tested using lmerTest (Kuznetsova et al., 2014) to determine pairwise significant differences where appropriate. Principal Component Analysis (PCA) was performed on the normalized qPCR dataset in order to analyze transcription patterns of all genes measured. PCA scores were calculated using the covariance matrix. Principal components 1 and 2 were determined to explain the majority of the variation in the data, using a Scree Test (Scree plot) as described in D'Agostino and Russell (2005).

3. Results

3.1. Physicochemical parameters

Dissolved oxygen, specific conductance, salinity, pH-value, and turbidity remained stable across all densities and replicates over the 24 h test period (Table 1). Light intensity was 40.3 lx (SD ± 7.9). Final ammonium concentrations across densities 7 to 56 ranged from 0.15 to 0.26 mg L^{-1}.

3.2. Cortisol

No significant differences between stocking densities for whole body cortisol were detected (F-value = 3.28, nested ANOVA P = 0.06). Cortisol levels were highest (up to 0.700 pg cortisol ·µg protein^{-1}) at lowest fish densities (7 and 14 fish per vessel) and decreased with increasing fish densities, with only 0.193 pg cortisol ·µg protein^{-1} measured at a density of 42 specimens per tank (Fig. 1). At a further increased fish density to 56 specimens per tank, cortisol levels again increased to a similar level as with 28 fish per tank. Normalized cortisol levels (mean and SD) are listed in Table 3. Detailed statistical information is listed in Supplementary Table S1.

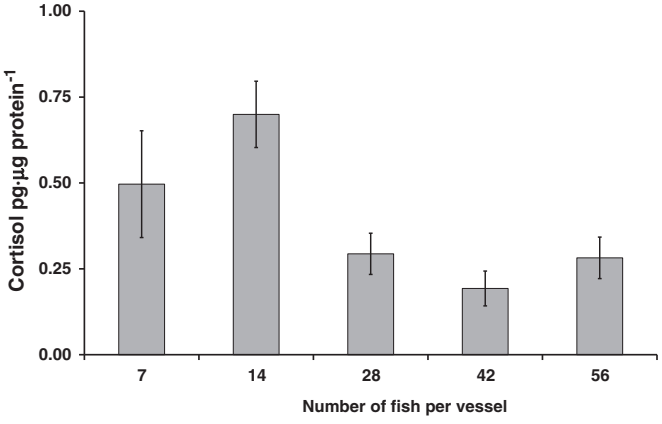

Fig. 1. Whole body cortisol levels for different Delta Smelt densities in pg cortisol ·µg protein^{-1}. Arithmetic mean across replicates (N = 3) with bars indicating standard errors.

Table 3
Relative transcript levels (Log-2 fold change) of genes and normalized whole body cortisol levels (pg cortisol ·μg protein^{-1}) used as biomarkers to determine stress levels in late larval Delta Smelt (*Hypomesus transpacificus*).

Density	Biomarker	11-Beta-HSD-1	POMC	GR2	MR1	11-Beta-HSD-2	GST	Cortisol
7	Mean	1.51	0.65	1.48	1.49	0.93	1.66	0.48
	SD	0.5	0.4	0.6	0.6	0.7	0.8	0.1
14	Mean	1.23	0.94	1.04	0.93	1.3	0.74	0.70
	SD	0.9	0.9	1.0	0.9	1.0	1.0	0.1
28	Mean	1.18	0.38	0.66	0.71	0.59	1.1	0.29
	SD	1.1	1.2	0.8	0.8	0.5	1.3	0.1
42	Mean	1.36	0.81	0.02	0.43	0.47	1.24	0.18
	SD	1.2	1.1	1.1	1.0	1.1	1.0	0.0
56	Mean	1.51	1.35	0.85	0.91	1.15	1.25	0.28
	SD	0.8	0.9	0.7	0.6	0.6	1.2	0.0

Abbreviations: GST: Glutathione-S-Transferase, POMC: pro-opiomelanocortin, MR1: Mineralocorticoid Receptor 1, GR2: Glucocorticoid Receptor 2, 11-Beta-HSD-1: 11-β-Hydroxysteroid-Dehydrogenase-Type 1, 11-Beta-HSD-2: 11-β-Hydroxysteroid-Dehydrogenase-Type 2, SD: standard deviation, SE: standard error.

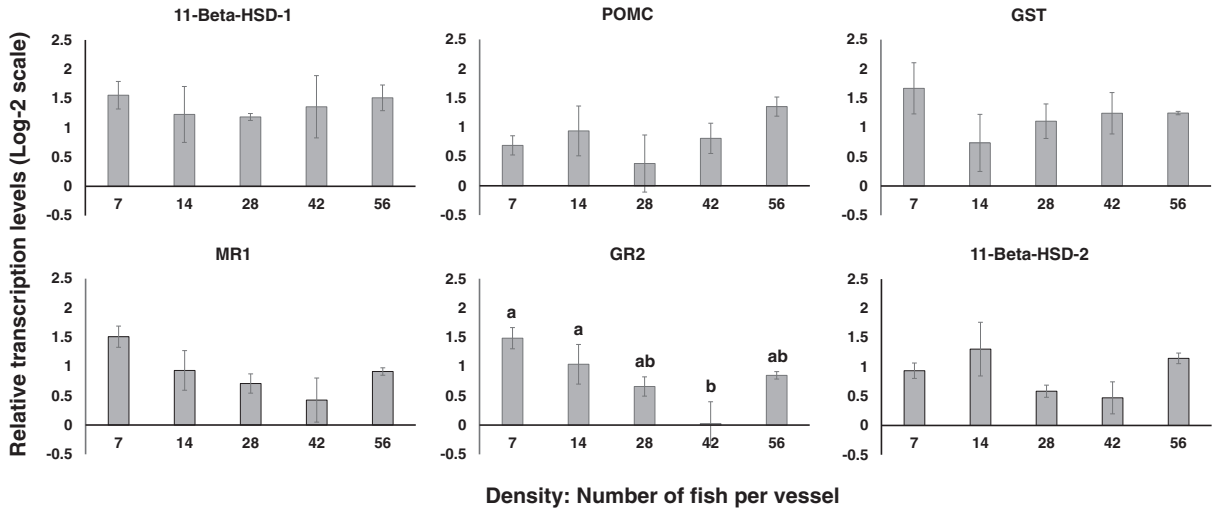

Fig. 2. Arithmetic mean Log-2 fold change (N = 3) in gene transcription per fish density level for all genes, relative to normalization factor calculated for two control genes. Bars indicate the standard errors. Letters indicate the significance differences (nested ANOVA, pairwise post hoc, significance level $\alpha = 0.05$) between density levels. Abbreviations: POMC (*pro-opiomelanocortin*), MR1 (*Mineralocorticoid Receptor 1*), GR2 (*Glucocorticoid Receptor 2*), 11-Beta-HSD-1 (*11-Beta-Hydroxysteroid-Dehydrogenase Type 1*), 11-Beta-HSD-2 (*11-Beta-Hydroxysteroid-Dehydrogenase Type 2*), GST (*Glutathione-S-Transferase*).

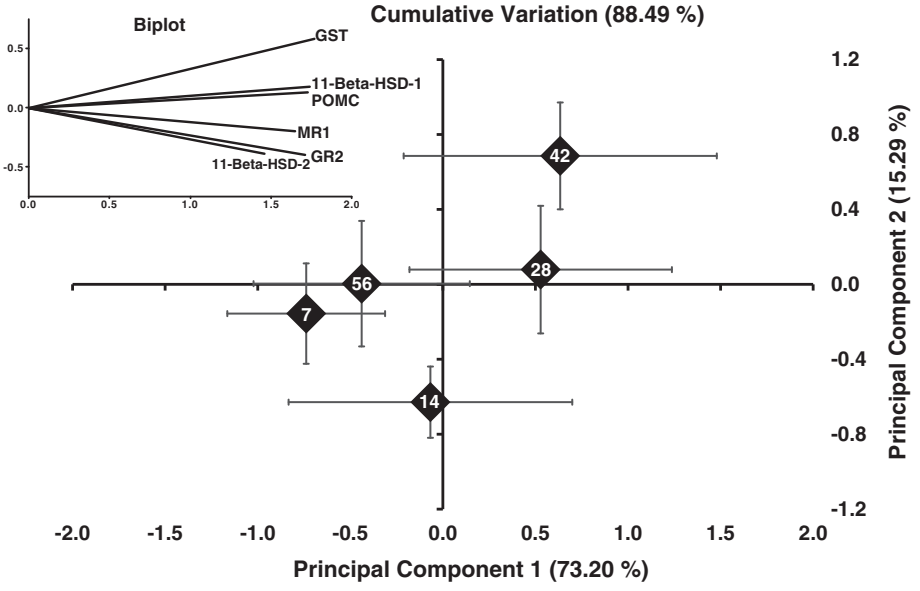

Fig. 3. Graphical depiction of the scores of principal components 1 and 2 for centroid graph for each treatment. Percentages give the amount of variation explained by the respective principal component. Numbers indicate the density levels (# of fish per vessel). Inset represents the respective biplot indicating genes driving the clustering. Legends of X-axis and Y-axis of the centroid graph are also applicable to the biplot. Abbreviations: POMC (*pro-opiomelanocortin*), MR1 (*Mineralocorticoid Receptor 1*), GR2 (*Glucocorticoid Receptor 2*), 11-Beta-HSD-1 (*11-Beta-Hydroxysteroid-Dehydrogenase Type 1*), 11-Beta-HSD-2 (*11-Beta-Hydroxysteroid-Dehydrogenase Type 2*), GST (*Glutathione-S-Transferase*).

3.3. Quantitative PCR

Significant differences were detected for GR2 between densities 7 and 42 fish (pairwise $P = 0.002$), and densities 14 and 42 (pairwise $P = 0.021$) (Fig. 2) with density 42 having the lowest expression levels and density 7 having the highest expression levels. Although not statistically significant, contrasting cortisol levels with mRNA expression levels of 11-Beta-HSD-2 revealed a similar pattern for densities 7 to 42 (Figs. 1 and 2). Furthermore, mRNA expression patterns of genes involved in cortisol production and regulation such as MR1, GR2, and 11-Beta-HSD-2 exhibited the lowest expression levels in densities 28 and 42, but were not significant, and POMC, a gene involved in initiating and stimulating the cortisol synthesis, had the lowest expression levels at density 28. Relative transcript levels of genes (mean and SD) are listed in Table 3. Detailed statistical information is listed in Supplementary Table S1.

3.4. Principal Component Analysis (PCA)

Principal component 1 explained 73.2% and principal component 2 15.3% of the variation among stress responsive genes, jointly describing 88.5% of the variation (Fig. 3). The respective biplot, plotted as an inset in Fig. 3, elucidates that genes MR1, POMC, and 11-Beta-HSD-1 loaded heavily on principal component 1, whereas 11-Beta-HSD-2, GR2, and GST loaded more on principal component 2. Densities of 28 and 42 fish clustered together, whereas the lowest assessed densities of 7 and 14 clustered more closely with 56.

4. Discussion

This study emphasizes that fish stocking density has a significant effect on fish physiological responses, and the importance of evaluating density stress, towards determining optimal conditions when planning an experimental design. Both biochemical (cortisol) and molecular (gene transcription) endpoints delivered consistent results as indicators of stress in stocking density experiments. The assessment of stress determined by using two mechanistic approaches can thus be used to establish a more comprehensive evaluation of density stress at multiple levels of biological organization, providing a higher level of confidence in subsequent experimental data.

Response pattern of genes utilized in this study, such as MR1, GR2, and 11-Beta-HSD-2 supported the findings of whole body cortisol levels being lower at densities 28 and 42, whereas others (POMC, GST, and 11-Beta-HSD-1) did not. Variability in responses between genes may be partly due to sex specificity, which has been described for juvenile rainbow trout (Momoda et al., 2007). Sex determination in Delta Smelt at this early life stage is, however, not feasible. The varying correspondences of genes with whole body cortisol levels are not surprising, because similar results between genes and their later product (proteins, hormones or metabolites) are not necessarily expected. Studies investigating the relationship between mRNA levels and their later products focus on proteins (Martyniuk et al., 2009, 2012) rather than on hormones/metabolites. Nonetheless, certain aspects of the mRNA–protein relationship also apply to mRNA–hormone relations, and several factors influence this relationship. Martyniuk et al. (2012) highlight that different kinetics and turnover rates, experimental lag times and sampling time points, as well as biological regulatory factors involved in transcription and translation contribute to the gene–protein relationship. One explanation for the low correspondence between genes POMC, 11-Beta-HSD-1, GST with cortisol patterns is a potential negative feedback loop mechanism which is common between protein and the respective gene, and also known in hormones (Martyniuk et al., 2009). While a single endpoint such as cortisol could potentially be sufficient to establish differences between treatments, our study highlights the benefits of having multiple endpoints, thus providing a greater overview of the mechanisms behind physiological stress responses.

The physiological stress response of late larval Delta Smelt to fish stocking densities followed a U-shaped curve. Results support our hypothesis for this pelagic species; with elevated stress levels observed at low stocking densities, as well as at elevated stocking densities. Both cortisol levels and gene transcription response indicated that the lowest stress levels were found at stocking densities in the range of 28 to 42 fish per 8 L vessel, suggesting this range as favorable within this test's conditions. These results are consistent with fish density values reported for optimal Delta Smelt culturing methods, which when converted to the same water volume used here, represent a stocking density range of 4 to 8 fish $\cdot L^{-1}$ for fish of similar age (41–80 dph) (Lindberg et al., 2013). Although Delta Smelt are not described as a schooling fish species, aggregations have been observed in the field (Bennett, 2005; Moyle, 2002). Aggregation likely provides the Delta Smelt with safety in numbers, sensory input, and protection from potential predators. This could explain the lower stress levels in treatments 28 and 42 fish per 8 L vessel compared to low and high stocking densities. Low stocking density might not allow for aggregation of sufficient fish to form a desirable sized group resulting in elevated stress.

Stress levels did not increase significantly in the highest stocking density treatments of 56 fish per 8 L vessel. This could stem from the fact that the highest tested density treatment of 56 fish was still within the stocking density range (30–60 fish per 8 L vessel) of the optimal culturing (Lindberg et al., 2013), and that water quality parameters remained in an acceptable range. Studies on stocking density effects in rainbow trout indicated that water quality affected certain physiological responses more than stocking density (Person-Le Ruyet et al., 2008).

Aside from water chemistry, additional factors such as water flow and physical characteristics of the experimental system (e.g., vessel shape and depth) play an important role for fish stocking density (Pickering, 1992). Often the applied density is only valid for the particular system used, in a particular hatchery/facility, and stocking densities must be modified before other systems are being utilized (Ellis et al., 2001). Numbers given for optimal cultural methods for Delta Smelt are determined for a recirculating holding system with freshwater supply, and biomedia filter, whereas the approach described herein used a static system.

Other factors such as fish species, age and size need to be considered for determining fish stocking density (Pickering, 1992). Differences in responses to stocking density levels have been observed in a number of species. Arctic charr, for example, were observed in several studies to have higher growth rates, greater length and weight, and lower aggressive interactions (Brown et al., 1992; Jørgensen et al., 1993; Wallace et al., 1988) and the highest feeding activity (Alanärä and Brännäs, 1996) at high densities. Further, this species was observed to have the lowest cortisol levels in higher densities compared to low densities (Vijayan and Leatherland, 1988). Pigfish (*Orthopristis chrysoptera*), a marine baitfish, also had higher survival rates in higher densities compared to low densities (DiMaggio et al., 2014). Other species have shown a different response to increased densities. For instance, in Senegalese sole (*Solea senegalensis*; Kaup 1858), the highest stress levels were found at high density treatments (Costas et al., 2008). These differences can primarily be explained by the distinct biology of each fish species; for example, fishes could be exhibiting specific territorial or schooling characteristics, significantly influencing density driven responses.

Although fish stocking densities have been studied intensively, mostly for aquaculture purposes, this study is one of few to specifically investigate the stocking density for an experimental protocol, and the first to conduct such evaluations using Delta Smelt. Fish stocking density has been discussed as an important factor in experimental biology, in the context of field experiments using cages as exposure vessels (Oikari, 2006). Improper fish stocking density can lead to enhanced stress levels and thus to a false or elevated stress response to the biological stressor tested, which in turn leads to misleading results and potentially misinterpretation of data. Therefore, it is of utmost importance to

adequately define the fish stocking density for the experimental protocol, with the aim of providing holding conditions with minimal stress, especially when measures of stress are being used as endpoints.

5. Conclusion

In this study we showed that fish stocking density has an effect on the physiological stress response of larval Delta Smelt. Both endpoints used in this approach delivered consistent results and are suitable for stress detection in fish stocking density experiments. The results highlight that taking stocking density in consideration as a potential confounding factor might benefit the quality of the experiment and quality of the stress response measured in the experiment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2015.07.005.

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