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Assessing oocyte development and maturation in the threatened Delta Smelt, *Hypomesus transpacificus*

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Abstract Delta Smelt is a pelagic annual fish species endemic to the San Francisco Estuary, which is protected under U.S. Federal and California State Endangered Acts. In this study we established criteria for scoring Delta Smelt maturity based on the histological features in ovary, as well as measurements of the female sex hormone 17β-estradiol (E2) from liver tissue using radioimmunoassay. We then analyzed the reproductive status of maturing females caught from November 2011 through April 2012 (2011 year-class) by histological examination, E2 quantification, and several common fish metrics including size of oocytes, gonadosomatic index, hepatosomatic index, and condition factor. Histological scoring revealed that Delta Smelt oocytes matured in February through April 2012. The presence of postovulatory follicles with immature oocytes (Stage 1 to 3) further suggests that Delta Smelt are iteroparous, capable of reproducing multiple times during the spawning season. Hepatic E2 concentration was significantly correlated with gonadosomatic index, hepatosomatic index, size of oocytes, and maturation stage. Histological examination of ovaries combined with measures of hepatic E2 and size of oocytes provide a powerful approach to evaluate reproductive performance and maturation timing for the imperiled Delta Smelt.

Keywords Delta Smelt · Reproduction · Maturity stage · Estradiol · Oocyte size

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Introduction

Understanding reproductive performance is the key foundation in estimating recruitment success of fish populations. Particularly, reproductive strategies and capabilities of female fish (such as timing of maturation, spawning area, fecundity, and egg quality) affect abundance and performance of offspring directly. Therefore these endpoints are widely investigated for conservation of fish populations in the wild (Billard and Lecointre 2001; Farley et al. 2013).

Maturation in fishes has been characterized by histological examination of gonadal tissue, or by quantification of specific reproductive hormones (Webb et al. 2002; Hajirezaee et al. 2012). The reproductive hormone, 17ß-estradiol (E2), is a primary female hormone involved in sex differentiation and sexual maturation in fishes, with concentrations in the bloodstream or serum being routinely measured in aquaculture for assessing maturity and timing of ovulation (Burke et al. 1984; Webb et al. 2002; Lubzens et al. 2010; Zhang et al. 2011).

E2 is primarily generated by ovarian granulosa cells, and released into the bloodstream where the hormone interacts with hepatocytes in the liver (Nagahama 1994; Lubzens et al. 2010). Upon stimulation by E2, hepatocytes begin to produce egg-yolk precursor, vitellogenin, as well as a constituent of egg shell, choriogenin (Nagahama 1994; Babin et al. 2007; Lubzens et al. 2010). The proliferation of immature oocytes, synthesis and accumulation of cortical alveoli, lipid droplets and subsequent vitellogenin are also regulated by E2 (Campbell et al. 2006; Miura et al. 2007; Lubzens et al. 2010). However, given its widespread use in aquaculture as a key indicator for assessing the onset of maturation, a number of publications that report E2 concentrations in wild-caught fishes is still limited due to the technical difficulties in collecting blood samples from live fish in fields. Although there are some publications for wild fish species (Sisneros et al. 2004; Fentress et al. 2006), the process of blood sampling is time consuming and not practical for processing a large number of fish, and is extremely challenging for sampling methods that require boats or research vessels.

Delta Smelt, *Hypomesus transpacificus*, is an annual pelagic fish species endemic to the Northern San Francisco Estuary currently protected under the U.S. Federal and California State Endangered Species Acts. It is semi-anadromous, predominantly distributed in the low salinity zone where salinity is lower than 7 psu at larval and juvenile stages during summer and fall (Station ID: 500 s), and starts migration to freshwater area in winter and early spring for spawning (Station ID: 700 s, Fig. 1., Moyle et al. 1992; Bennett 2005). Most adults die after spawning and complete their life cycle in 1 year (Moyle et al. 1992; Bennett 2005).

The maturity of female Delta Smelt is assessed by gross examination of ovaries based on their appearance (color, size, etc.), which is currently employed in the routine monitoring of Delta Smelt during the spawning season (Bennett 2005). Although this method is quick and practical, not requiring any special equipment, gross examination is qualitative and somewhat subjective, as well as characterizes maturation as a series of discrete stages rather than a continuous process. In addition, our understanding of Delta Smelt reproduction is still limited; for example, eggs which adhere to substrate have never been found in the wild (Bennett 2005). Thus a better understanding of the onset and timing of maturation is important especially given its primarily annual life cycle as well as for monitoring the reproductive health and development of rehabilitation options for this imperiled species.

In this paper, we describe key histological characteristics in ovaries and measurement of hepatic E2 concentrations to accurately determine the onset and status of maturation in wild-caught Delta Smelt. The methodologies described in this study provide more accurate and quantifiable assessments of the maturation process for Delta Smelt, and thus key information on the reproductive status and timing of spawning for this imperiled species.

Materials and methods

Study area

The San Francisco Estuary is the largest estuary on the west coast of the United States, providing vital water for human consumption as well as habitats for native fish species (Nichols et al. 1986; Brown et al. 2013). Figure 1 indicates the sampling stations in this study; encompassing Benicia (Station ID: 400 s) to the Sacramento River (Station ID: 700 s) and to the San Joaquin River (Station ID: 800 s). The hydrology and salinity of the San Francisco Estuary is highly affected by freshwater inputs from the Sacramento and San Joaquin River, and exports for agricultural use (Bennet et al. 2002).

Fish sampling

Sub-adult (November through December in 2011) and adult Delta Smelt (January through April in 2012), collected during the ongoing fish monitoring surveys were provided by the California Department of Fish and Wildlife. After capture, Delta Smelt were individually wrapped in aluminum foil and assigned an identification tag with survey name, station ID, and date of collection information in the field. Each fish was flash-frozen and stored in liquid nitrogen, and then transported to the Aquatic Health Program at the University of

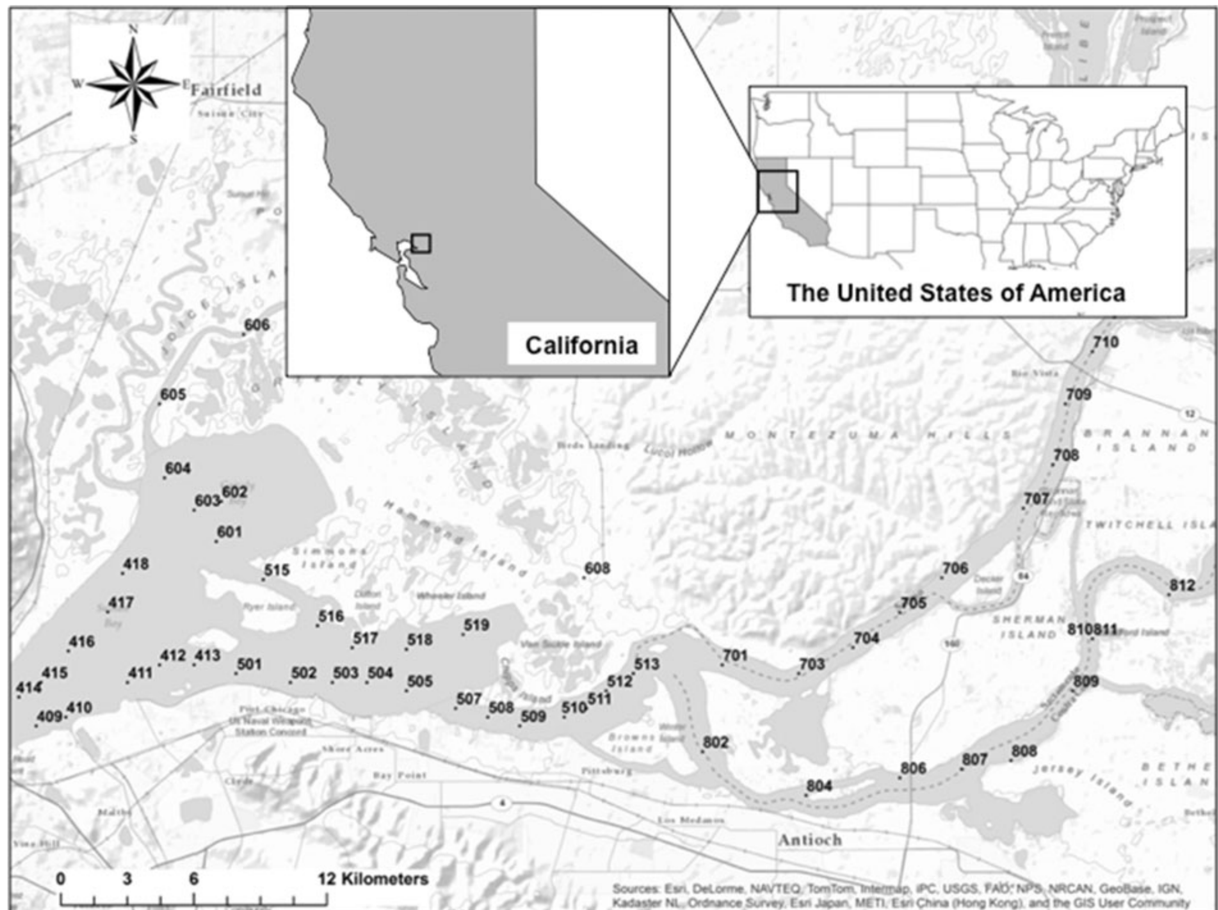

Fig. 1 Geographical location and the sampling stations for the ongoing Delta Smelt monitoring program operated by the California Department of Fish and Wildlife (CDFW). Not all the sampling stations are depicted due to the space limitation. The map was created by ArcMap ver. 10.1 software (http://www.esri.com/software/arcgis) with the GPS coordinates provided by CDFW

California, Davis. During the field sampling, ancillary data on water quality, such as water temperature, salinity, and turbidity were also recorded at each sampling site.

All female Delta Smelt caught in November ($n=20$) and December ($n=64$) 2011 were examined while in 2012 subsampling was employed in January through April due to the increased catch numbers. The number of female fish used for the analysis in 2012 were as follows: January, $n=70$; February, $n=36$; March, $n=40$; and April; $n=63$. While still frozen, fork length and weight were recorded, and then livers and ovaries were dissected when partly defrosted. The ovary and liver tissues were then weighed and partitioned for the analyses. One ovarian partition was preserved in 10 % buffered formalin for histology and the remaining was stored in −80 °C for future use. Likewise, one liver partition was fixed in 10 % buffered formalin for histology, whereas the remaining was stored in −80 °C before being assessed for E2 quantification.

Histology for scoring maturity of fish and measuring size of oocytes

Ovaries fixed in 10 % buffered formalin were embedded in paraffin blocks using a HISTOEMBEDDER, and sectioned using a Leica 2155 microtome to a width of 3 μm (Leica Microsytems, Buffalo Grove, IL). Each section was mounted onto a microscope slide, and was left to dry overnight. After drying, each slide was stained by hematoxylin and eosin. Histological sections of ovaries were examined under light microscopy at a magnification of 100X and were classified to one of the maturity stages based on a

modified method adapted from Mager (1996) and Dadzie (2007) (Fig. 2, Table 1). The most advanced oocytes with high prevalence (>90 %) were used to classify the maturity stage of fish. Cryopreservation of Delta Smelt in liquid nitrogen caused artefacts in the internal structure of oocytes, particularly at Stage 3 and 4, however the key histological features for staging were still preserved and maturity level was accurately assessed (Teh et al. 2016).

To ensure an accurate measurement of oocyte dimensions, only oocytes with most advanced stage, showing a visible nucleus transection, were selected for the analysis (Cernadas et al. 2008). The cross-sectional area (mm^2) of fish oocytes was obtained using ImageJ software (http://rsbweb.nih.gov/ij/) by setting a scale with micro-ruler, tracing the oocyte rim manually, and then calculating the cross-sectional area of each oocyte using computational equation in the software. The average oocyte size and standard deviation were obtained by measuring at least 10 oocytes randomly selected from each fish.

Radioimmunoassay (RIA) for hepatic 17β-estradiol (E2) quantification

Hepatic E2 concentrations were measured by radioimmunoassay as follows; frozen liver tissues were weighed (ca. 4 mg) and 500 μl of ice-cold 1X phosphate buffered saline (PBS) was added to each tissue sample. Tissues in PBS were sonicated using Misonix XL2000 Ultrasonic Homogenizer (Fisher Scientific, Hampton, NH), then centrifuged at 12,000 rpm for 10 min. Supernatants (ranging 350–400 μl) were carefully transferred into clean borosilicate glass tubes, avoiding collection of the top lipid layer and bottom insoluble proteins. Small portions (40 μl) of the liver homogenates were saved for subsequent Lowry assay to determine protein concentrations (Lowry et al. 1951). All the samples were kept cold (4 °C) during sonication and centrifugation steps. Diethyl ether (2 ml) was added to the each liver homogenate and mixed vigorously by vortex mixer for a minimum of two minutes to maximize the recovery of E2. The homogenates were placed in dry ice-ethanol bath to

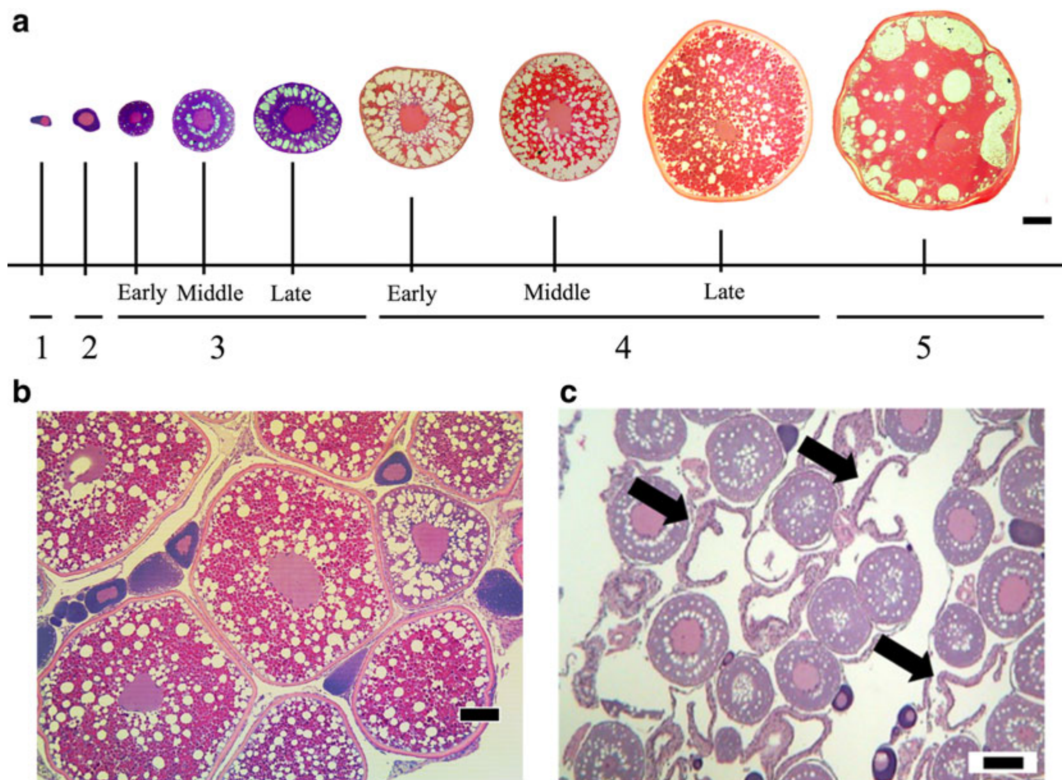

Fig. 2 Histological images of Delta Smelt oocytes. Histological images of individual oocyte in each stage are shown in Panel **a**. An example of histological image taken from Delta Smelt ovary as scored as Stage 4 Late is shown in Panel **b**. Panel **c** demonstrates a Delta Smelt ovary of post-spawner (Stage 6) with postovulatory follicles (indicated by the *black arrows*). The photos were taken from fish without cryopreservation. Scale bars: 100 μm

freeze the bottom aqueous layer, and the top diethyl ether layer containing E2 was transferred into a new clean borosilicate glass tube. The diethyl ether was volatilized under air stream in a chemical fume hood overnight. The extracted E2 was subjected to quantification using competitive RIA available at the Clinical Endocrinology Lab, University of California, Davis.

The RIA was performed as follows; the dried samples were resuspended in 300 μl RIA buffer (1xPBS with 0.1 % gelatin). Rabbit anti-E2 polyclonal anti-sera produced by the Clinical Endocrinology Lab (100 μl) was subsequently added to each sample at final dilution of 1:250,000 (Shille et al. 1979). After further addition of 100 μl of hydrogen-3 (^{3}H)-labeled estradiol (9000 cpm), samples were vortexed and incubated at 4 °C overnight. Charcoal-dextran buffer (0.5 % charcoal and 0.05 % dextran, 500 μl) was added to each tube, incubated for 20 min at 4 °C, then centrifuged at 2000×*g* for 20 min. The supernatant was poured into scintillation vials and the signal of unbound radioactive ^{3}H-labeled estradiol was measured on a liquid scintillation counter (Tri-carb 2000, PerkinElmer, Waltham, MA), and the E2 concentrations were calculated based on the standard curve (Rodbard 1974). The reaction was performed without replicates due to the limitation of tissue size.

A Lowry assay was used to measure total protein concentrations for each liver homogenate in order to standardize E2 concentrations (Lowry et al. 1951). The assay was performed using a commercially available kit following the manufacturer's instruction with 1:2 serial dilutions of bovine serum albumin standards, ranging 0.3125 to 40 mg/ml (DC Protein Assay, BioRad, Hercules, CA). The reaction was carried out in 96-well plates in triplicate. Averages from three replicates were taken for the analysis, and the results of E2 concentration were expressed as picograms of E2 per one milligram of liver protein. E2 or protein concentrations that did not fall in the dynamic range of the assays were not included in the analysis (E2 RIA <2.5 pg, Lowry assay <0.3125 mg/ml). E2 concentrations for each maturity stage were shown in boxplots with median indicated by the black center-line because the dataset was not normally distributed as assessed by Shapiro-Wilk Test (Shapiro and Wilk 1965).

Statistical analysis

Comparisons of E2 concentrations in Stage 3 and 4 (without sub-classification) were performed using Wilcoxon Signed-Rank Test (Wilcoxon 1945). Spearman's rank correlation test was used to determine significant relationships among the various indices: condition factor (CF), hepatosomatic index (HSI), gonadosomatic index (GSI), maturity stage, E2 concentration, and size of oocytes (Spearman 1904). CF, HSI, and GSI were obtained by the following formula:

$$CF = \frac{\text{Fish body weight(g)}}{[\text{Fish fork length (cm)}]^3} \times 100$$

$$HSI = \frac{\text{Liver weight(g)}}{\text{Fish body weight(g)}} \times 100$$

$$GSI = \frac{\text{Gonad weight(g)}}{\text{Fish body weight}(g)} \times 100$$

All the indices were tested for normal distribution and possible outliers. Non-parametric methods were used to analyze the data when collected data were not normally distributed. All the statistical analyses were performed using STATISTICA ver. 8.0 (StatSoft, Tulsa, OK).

Results

Based on the histological features, maturity of Delta Smelt oocytes was categorized into one of the six major stages; immature stage (Stage 1 and 2), cortical alveolus stage (Stage 3), vitellogenic stage (Stage 4), final maturation stage (hydration stage, Stage 5), and post-spawner (Stage 6) as shown in Fig. 2 and Table 1. The oocytes in Stage 3 and 4 were further sub-classified into three substages (Early, Middle, and Late) according to the abundance of cortical alveoli (Stage 3) and egg yolk bodies (Stage 4), respectively.

Delta Smelt showed shifts of oocyte maturation in concordance with the time of sampling (Fig. 3, Panel a). Delta Smelt in Stage 3 (Early, Middle and Late) were dominant from November 2011 through January 2012, accounting for more than 50 % of the females collected in each month. This trend significantly changed in February and afterwards, where Stage 4 (Early, Middle and Late) dominated the populations (>70 %). A number of post-spawning Delta Smelt (Stage 6) were observed in March and April, 25 and 33 %, respectively. Ovaries of

Table 1 Developmental stages of Delta Smelt oocytes. Maturity of oocytes was categorized into one of the six major stages with sub-stages for Stage 3 and 4 based on the histological features. The scoring criteria were adopted from Mager (1996)

Stage	Sub-stage	Description
1		Immature Stage: primordial oocyte (PMO) with centrally located nucleus, containing a single nucleolus
2		Immature Stage: primary growth oocyte (PGO) with a larger nucleus and multiple nucleoli located at the periphery of the nucleus
3		Cortical Alveolus Stage: characterized by enlarged oocytes with cortical alveoli present in the cytoplasm
	Early	Small cortical alveoli lining the periphery of the cytoplasm
	Middle	Larger cortical alveoli become present in the cytoplasm
	Late	Cortical alveoli increase in size and quantity, and become present throughout the cytoplasm
4		Vitellogenic Stage: characterized by a dramatic increase in size with cytoplasm containing eosinophilic yolk bodies
	Early	Small eosinophilic yolk bodies can be seen forming in the cytoplasm
	Middle	The number of yolk bodies increases
	Late	Enlarged yolk bodies are present throughout the cytoplasm, interspersed with cortical alveoli. The germinal vesicle starts to migrate toward double chorion layer
5		Final Maturation (Hydration) Stage: characterized by hydration and the fusion of yolk bodies into a homogenous yolk mass, forming yolk plate
6		Post-Spawner: characterized by presence of postovulatory follicles as a result of ovulation (Fig. 2, Panel c). Primordial (Stage 1) and primary growth oocytes (Stage 2), and oocytes with cortical alveoli (Stage 3) are also present.

these post-spawned fish showed postovulatory follicles concurrently with the next clutch of oocytes developing at Stage 3 (Fig. 2, Panel c). Size of oocytes, GSI, E2 concentrations, and HSI increased in concordance with the time of sampling (Fig. 3, Panels b, c, d, and e) while CF remained relatively constant (Fig. 3, Panel f).

The E2 concentrations remained relatively low until Stage 4 Early (median = 10.8 pg/mg), followed by prominent increase in Stage 4 Middle (median = 20.7 pg/mg) and Late (median = 32.2 pg/mg), and returned to a basal level after spawning classified as Stage 6 (post-spawner) (Fig. 4, Panel a). The E2 concentrations for the combined Stage 4 fish (median = 19.5 pg/mg) was significantly higher than those for the combined Stage 3 fish (median = 6.0 pg/mg) as shown in Fig. 4, Panel b (P value < 0.01).

The oocytes enlarged progressively as fish matured; prominent increase at Stage 4 Late (median = 0.3796 mm^2) and reaching plateauing at Stage 5 (median = 0.3625 mm^2, Fig. 5). This was followed by drastic drop in Stage 6 (post-spawners) to the sizes similar to Stage 3 Late (Stage 3 Late: median = 0.04586 mm^2, Stage 6: median = 0.04900 mm^2). This pattern is coinciding with dynamic changes of hematic E2 concentrations (Figs. 4 and 5).

Spearman's rank correlation coefficient revealed that E2 concentration was positively correlated with the somatic indices (CF, HSI, and GSI), categorical maturation stage as well as size of oocytes (Table 2). In particular, the E2 concentration was highly correlated to HSI, GSI, and size of oocytes ($\rho > 0.60$). Likewise, high correlation values were observed between size of oocytes and somatic indices, HSI ($\rho = 0.76$) and GSI ($\rho = 0.94$).

Discussion

To assess reproductive performance of female Delta Smelt in the wild, we established methods for assessing maturity level based on histological features and hepatic E2 concentrations. The histological analysis provides baseline information on maturation level of wide range of oocytes from immature to maturing oocytes. Measuring hepatic E2 concentrations at particular maturation stage, especially late vitellogenic stage, possibly imply timing of ovulation. This unique approach with multiple reproductive endpoints provides powerful tools for understanding the maturation and reproductive performance of the imperiled Delta Smelt.

Application of histological technique for assessing maturity level of oocytes provides greater precision than traditional techniques such as gross examination and microscopy (Murua et al. 2003). Although gross examination is more practical especially in the field, the method is not feasible to distinguish oocytes at final maturation stage (hydration stage, Stage 5) from vitellogenic stages (Stage 4) because oocytes at the two stages are apparently identical (data not shown). Stage 5

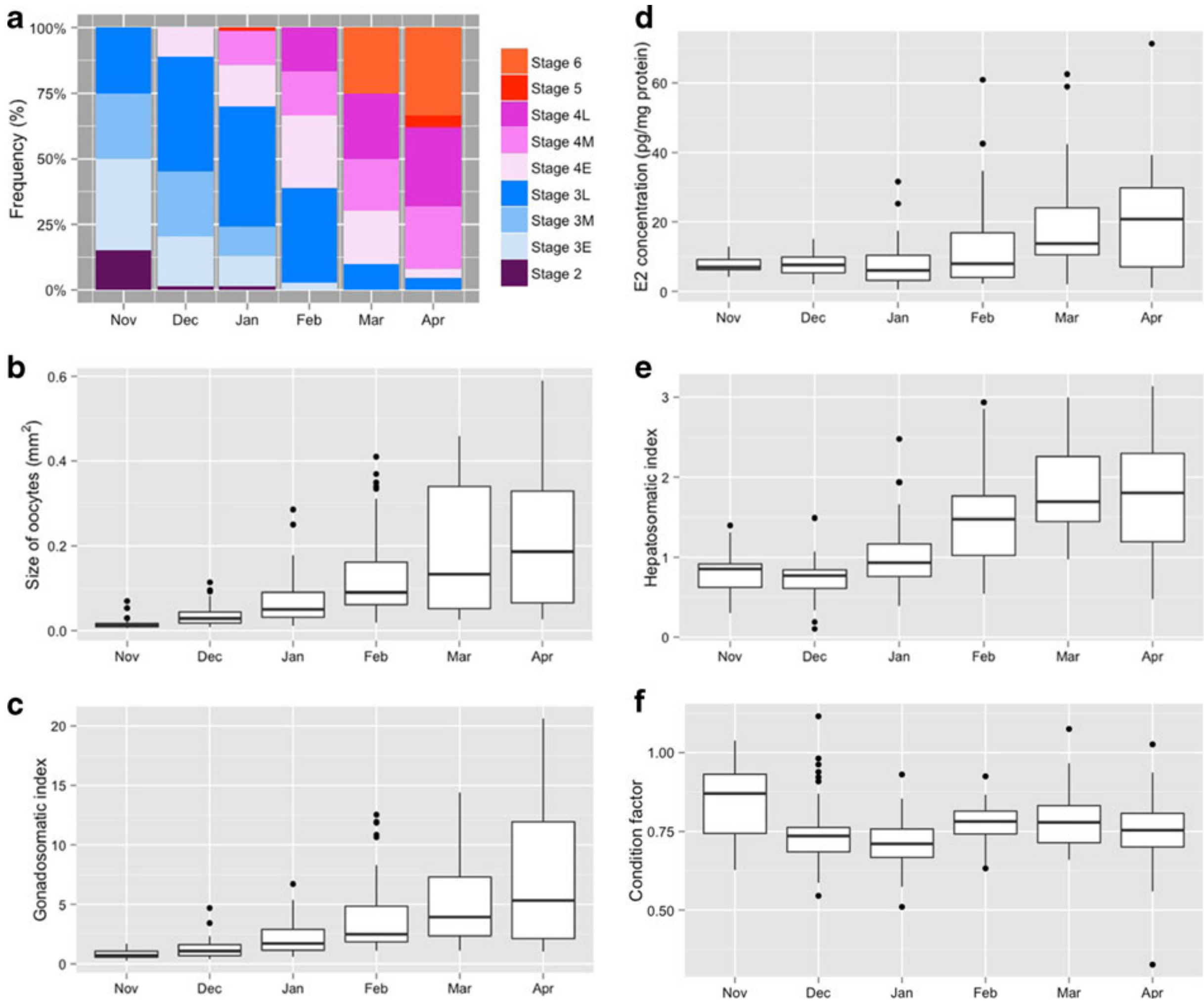

Fig. 3 Monthly changes in maturity and morphometric indices for 2011 year-class of Delta Smelt, caught from November 2011 through April 2012. Female Delta Smelt at sub-adult through adult stages (Nov: $n=20$, Dec: $n=64$, Jan: $n=70$, Feb: $n=36$, Mar: $n=40$, and Apr: $n=63$, total 293 fish) were subjected to the analyses for scoring maturity (Panel **a**), measuring size of oocytes (Panel **b**), gonadosomatic index (Panel **c**), E2 concentrations (Panel **d**), hepatosomatic index (Panel **e**), and condition factor (Panel **f**). Maturity of fish was scored based on the histological features of oocytes as described in Fig. 2 and Table 1. The upper and bottom hinges of the *boxes* indicate 75 and 25 percentile. The *whiskers* indicate minimum and maximum values observed, and outliners are depicted by *dots*

is characterized by final maturation (hydration) and its retention time seems to be very short as indicated by very limited number of Stage 5 fish observed in this study (Fig. 3a). In anchovy and seatrout, the final maturation and spawning can be completed within 24 h upon stimulation by the proper biotic (hormonal) or abiotic cues (Hunter and Macewicz 1985; Brown-Peterson et al. 1988). The retention time of Delta Smelt at Stage 5 is still unknown, however, if the retention time of Stage 5 is short like other fish species, geographical distribution of Delta Smelt at Stage 5 possibly indicates their spawning locations in the San Francisco Estuary, which is currently unknown despite of its importance for protection.

Histological examination further provides us tools to investigate the Delta Smelt maturity shifts for understanding the timing of maturation and for predicting possible spawning time of cohorts. We propose to use Delta Smelt at Stage 4 Late to predict reproductive performance of year-classes. Stage 4 Late is characterized as the end of vitellogenic stage where the oocytes dramatically increase in size, accumulate yolk bodies and form a vitelline envelope (Fig. 2a, b). The oocytes at

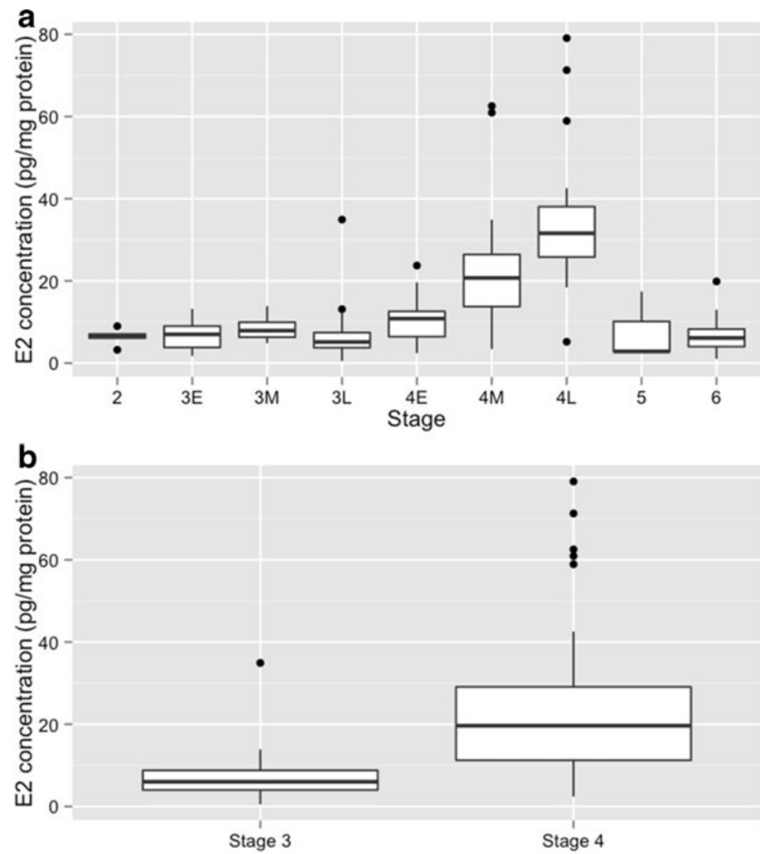

Fig. 4 Concentrations of hepatic17ß-estradiol (E2) for female Delta Smelt in relation to different maturation levels. E2 concentration was expressed as picogram per milligram of liver protein. The boxplots were generated for all the stages with sub-classification (**a**) and for combined Stage 3 and 4 (**b**). The upper and bottom hinges of the *boxes* indicate 75 and 25 percentile. The *whiskers* indicate minimum and maximum values observed, and outliners are depicted by *dots*

Stage 4 Late can be artificially inseminated by stripping from female fish (Lindberg et al. 2013), indicating oocytes at the stage are fully capable of fertilization and have less possibility to undergo atresia due to environmental stress whereas the fate of oocytes at earlier stages such as Stage 4 Middle is unpredictable (Lubzens et al. 2010). We are currently using abundance of Delta Smelt at Stage 4 Late and their fecundities to estimate reproductive capability of an entire year-class, which can be further used as a baseline for assessing groups of fish collected from different years.

Although measuring hepatic E2 concentration is uncommon, the results obtained in this study demonstrate that liver can be an alternative biological material for measuring E2 concentrations. Liver is the primary tissue that produces precursor proteins for egg yolk

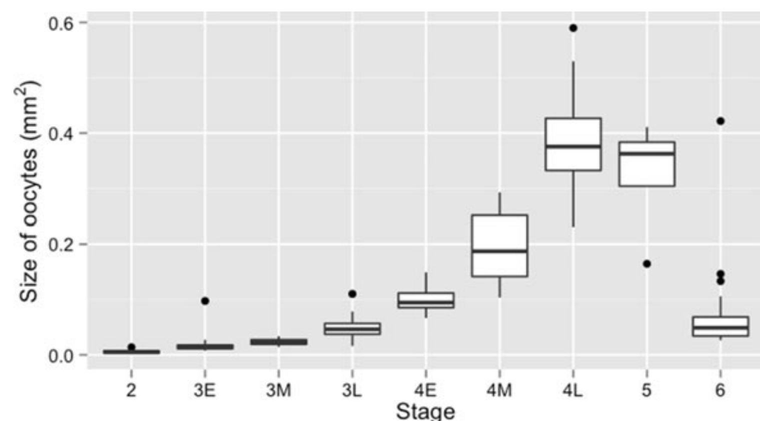

Fig. 5 Size of oocytes for female Delta Smelt in relation to different maturation levels. The upper and bottom hinges of the *boxes* indicate 75 and 25 percentile. The *whiskers* indicate minimum and maximum values observed, and outliners are depicted by *dots*

Table 2 Spearman's rank correlation coefficient for Delta Smelt indices (Spearman's ρ). A total of 213 fish were analyzed for the comparison of the indices (casewise deletion for missing data)

Indices	CF	HSI	GSI	Stage	E2
Condition Factor (CF)					
Hepatosomatic Index (HSI)	0.31781				
Gonadosomatic Index (GSI)	0.37482	0.72590			
Stage[a]	−0.00012	0.42382	0.32523		
Estradiol (E2)	0.32218	0.68441	0.67966	0.19336	
Size of oocytes	0.37733	0.76417	0.94087	0.37473	0.68866

[a]Categorical data

(vitellogenin) and egg-shell (choriogenin) upon stimulation by E2 (Nagahama 1994; Babin et al. 2007; Lubzens et al. 2010). In our study, the characteristic changes of hepatic E2 concentrations were observed; the hormone level increased significantly as Delta Smelt became more reproductively mature at Stage 4 Late, and decreased back to basal level in later stages (Stage 5 and 6) (Fig. 4a). Stage 5 ovarian follicles switch from the production of E2 to the synthesis of the maturation inducing hormones, such as 20β-dihydroprogesterone, 17α,20β-dyhydroprogesterone, and 17α,20β,21-trihydroxy-4-pregnen-3-one, for the final maturation step of hydration (Dahle et al. 2003; Nagahama and Yamashita 2008). This pattern has been well described by other researchers for various types of fish species including Atlantic Cod, English Sole, European Sea Bass and Huchen (Johnson et al. 1991; Dahle et al. 2003; Miura et al. 2007; Rocha et al. 2009). Furthermore, E2 may provide us more insight into reproductive health, especially for exposure by contaminants. Exposure of female fish to noxious chemicals (pollutants, toxins) or environmental stress (low oxygen, insufficient prey availability, or poor water quality) can affect E2 levels in fish plasma, resulting in reproductive failure. Significant reduction of GSI and plasma level of E2 was reported by 1 month exposure of Atlantic Croaker to lead and benzo[a]pyrene (Thomas 1988). Johnson et al. (1997) documented reproductive dysfunctions such as inhibition of gonadal development, depression of plasma E2 level, and reduced spawning success in the English Sole collected from an area polluted with aromatic hydrocarbons or polychlorinated biphenyls.

In this study, we established methodologies for assessing maturity of Delta Smelt based on histological and hormonal examinations. Although it's not major focus in this paper, size of oocytes, which can also be obtained from histological sections, likely represents quality of eggs during spawning as there is a correlation between egg size and size of fry in other fish species (Springate and Bromage 1985). This improved approach with multiple reproductive endpoints provides more accurate and quantifiable assessments of maturity status of Delta Smelt and permits us to further understand spatiotemporal reproductive status and performance of the endangered fish species.

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