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Expression of *HOX* Cluster Genes in Early Larval Development of Sea Urchin *Strongylocentrotus intermedius* (Echinodermata, Echinoidea)

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Abstract—Expression of 11 genes of the *Hox* cluster (*SiHox1*, *2*, *3*, *5*, *6*, *7*, *8*, *9/10*, *11/13a*, *11/13b*, and *11/13c*) was assessed in the sea urchin *Strongylocentrotus intermedius* at early developmental stages, including the blastula (13 h post fertilization (hpf)), gastrula (35 hpf), prism (46 hpf), and pluteus (4 and 9 days post fertilization (dpf)) stages. Expression of *SiHox7*, *11/13b*, and *11/13c* was observed at the blastula stage; early activation of *11/13c* was detected for the first time in regular sea urchins. The expression level was very low at the gastrula and prism stages. The pluteus stage showed a significant increase in expression of all *Hox* cluster genes. Deviations from temporal collinearity of *Hox* gene expression in echinoderms are discussed.

Keywords: real-time PCR, blastula, gastrula, prism stage, pluteus

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Regulatory genes of the *Hox* cluster determine the sequential establishment of body structures along the anteroposterior axis in the animal [1, 2]. Spatial and temporal collinearity characterizes expression of the 15 *Hox* genes in the basal deuterostome group Cephalochordata. That is, the genes start expression consecutively, rather than simultaneously, from the 3' to the 5' end of the *Hox* cluster and from the anterior to the posterior body segments of the developing embryo [3, 4]. However, certain deviations from collinearity in expression of the *Hox* genes are observed in various groups of Deuterostomia, as well as various changes in the composition of the *Hox* cluster. In regular sea urchins, the *Hox* cluster includes 11 genes: *Hox1*, *2*, *3*, *5*, *6*, *7*, *8*, *9/10*, *11/13a*, *11/13b*, and *11/13c* [5, 6], however, data on the gene order within the *Hox* cluster are discrepant; e.g., a translocation of the anterior gene group including *Hox1*, *2*, and *3* from the 3' to the 5' end of the *Hox* cluster has been described. A similar gene set of the *Hox* cluster has been observed in Crinoidea [7]. Various additional reductions of the *Hox* cluster have been detected in the other classes of Echinodermata [4]. Members of the phylum Hemichordata, which is a sister group to Echinodermata, have a *Hox* gene set similar to that in sea urchins [8, 9]. Apart from changes in the order of genes in the *Hox* cluster, deviations from temporal collinearity in *Hox* gene expression have been observed in certain echinoderms, i.e., expression of the *Hox* genes starts from the central or posterior, rather than anterior, gene group of the cluster [10, 11]. Data on the organization of the *Hox* cluster and the order of expression of the regulatory *Hox* genes in various groups of animals are accumulating gradually, being subject to revision and giving origin to new evolutionary hypotheses. The objectives of our work was to identify the genes of the *Hox* cluster and to assess their expression during early larval development in the indirect-developing regular sea urchin *Strongylocentrotus intermedius*.

The following early delopmental stages were examined in *S. intermedius* (Fig. 1): the blastula (13 h post fertilization (hpf)), gastrula (35 hpf), prism (46 hpf), and pluteus (4 and 9 days post fertilization (dpf)) stages. All experiments with fertilization and development were carried out at the Vostok Marine Biological Station, A.V. Zhirmunsky National Scientific Center of Marine Biology (NSCMB), Far Eastern Branch of Russian Academy of Sciences. Sea urchins were collected in the Vostok Bay of the Sea of Japan. The sea urchins were kept in tanks with running aerated water and washed with UV-treated filtered seawater two or three times prior to experiments. Spawning was induced by injecting 1–2 mL of 0.5 M potassium chloride into the Aristotle's lantern cavity. Larvae were obtained via artificial fertilization and cultured at 18°C.

Total RNA was extracted from *S. intermedius* eggs and early larvae with a Yellow Solve kit (Clonogen,

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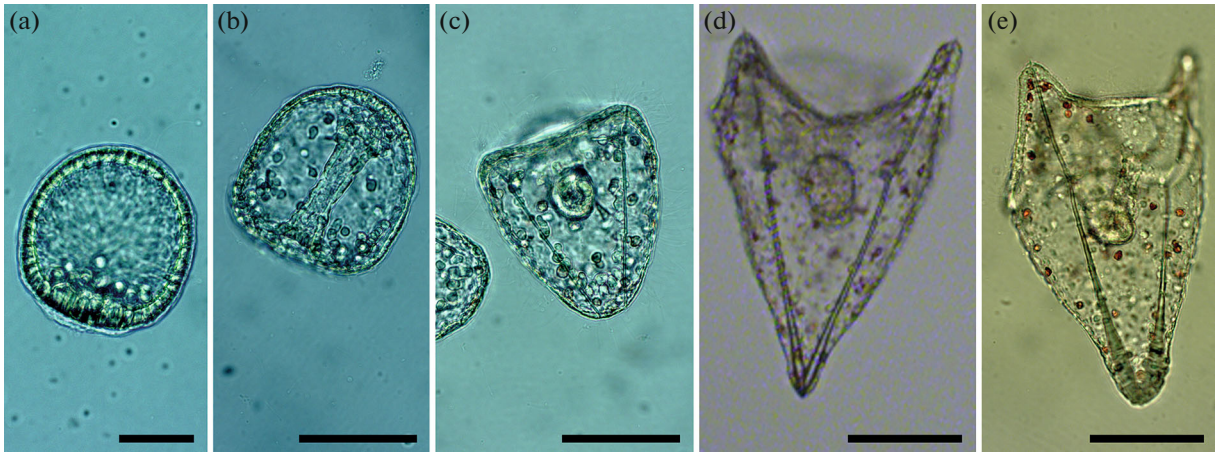

Fig. 1. *Strongylocentrotus intermedius* early developmental stages examined in this study: (a) blastula, 13 hpf; (b) gastrula, 35 hpf; (c) prism stage, 46 hpf; (d) pluteus, 4 dpf; and (e) pluteus, 9 dpf. Bar, 50 μm.

St. Petersburg, Russia). The total RNA concentration was estimated by spectrophotometry (Shimadzu, Japan). Complementary DNA (cDNA) was synthesized using a reverse transcription kit (Sileks, Moscow, Russia) and 1–3 μg of isolated total RNA. Reverse transcription–polymerase chain reaction (RT–PCR) was carried out in the reaction mixture (50 μL), which contained a 1× RT buffer, 0.24 mM each dNTP, 0.2 μM oligo-$(dT)_{15}$ as a primer, and 200 units of MMLV reverse transcriptase. The reaction was carried out at 37°C for 2 h. The product (0.5 μL) was amplified via PCR. To standardize the samples in terms of cDNA quality and amount in each real-time PCR (qRT-PCR) experiment, normalization was carried out using the actin (GenBank DQ229162) and ubiquitin (GenBank LOC754856) genes of *S. intermedius* as an endogenous control. Data were collected in five independent experiments. qRT-PCR primers directed to the actin and ubiquitin genes were as described previously [12].

Sequencing was performed using an ABI 3130 genetic analyser (Applied Biosystems, United States) at the Federal Research Center of East Asian Terrestrial Biodiversity. Each transcript was sequenced at least three times.

To theoretically design the specific qRT-PCR primers directed to the *S. intermedius Hox* genes, the nucleotide sequence of the *Hox* gene cluster was analyzed in the related species *S. purpuratus* [13, 14]. All data and respective search and visualization tools are available through the SpBase public sea urchin genome database (http://www.spbase.org/SpBase/rnaseq/) and the NCBI BioProject database (http://www.ncbi.nlm.nih.gov/bioproject), bioproject no. PRJNA81157. Sequencing data were used to design the specific qRT-PCR primers via NCBI Primer Blast software.

Expression levels of the *S. intermedius Hox* genes were estimated individually for each gene and measured in relative units (rel. units), considering the amplification level of the respective gene in unfertilized eggs as the conditional value "1". Quantitative estimates of the expression levels were obtained for all of the 11 *Hox* genes at early developmental stages of *S. intermedius* (Fig. 2).

At the blastula stage (13 hpf), *Hox* gene expression levels were mostly lower than in eggs (lower than 1 rel. unit). The *SiHox7*, *11/13b,* and *11/13c* genes were exceptions; their expression increased (Fig. 2). In the other sea urchin species S. purpuratus, high-level expression of two *Hox* genes, *SpHox7* and *11/13b*, at the blastula stage (24 hpf) has been reported [10, 15, 16]. Early activation of all of the three genes (*PjHox7*, *11/13b,* and *11/13c*) at the blastula stage (6–10.5 hpf) has been detected in the direct-developing sea urchin *Peronella japonica* [11].

At the gastrula (35 hpf) and prism (46 hpf) stages, extremely low expression levels were observed for the *SiHox* genes. Only *SiHox11/13a* slightly increased in expression at the gastrula stage, while expression of all other genes continued to decrease (Fig. 2). According to published data on *Hox* gene expression in *S. purpuratus*, the neighbor *SpHox11/13b* increases in expression during gastrulation (21–36 hpf) in the species [15] and decreases substantially in the late gastrula (48 hpf) [10]. However, our study showed that *SiHox11/13b* dramatically decreased in expression as early as 35 hpf in *S. intermedius*, although its expression slightly increased at the prism stage, still remaining far lower than in the blastula (Fig. 2). In *P. japonica*, *PjHox9/10* starts expression at the gastrula stage (13–15 hpf) in addition to already active *PjHox7*, *11/13b*, and *11/13c*. Expression of *PjHox1*, *7*, *8*, *9/10,* and *11/13b* is observed at the prism stage (18–21 hpf), while *PjHox11/13c* expression stops and is not restored later, at least during early development [11].

The pluteus formation (4 dpf) was associated with an abrupt activation of all 11 genes of the *Hox* cluster in *S. intermedius* (Fig. 2). Later (9 dpf), we observed an

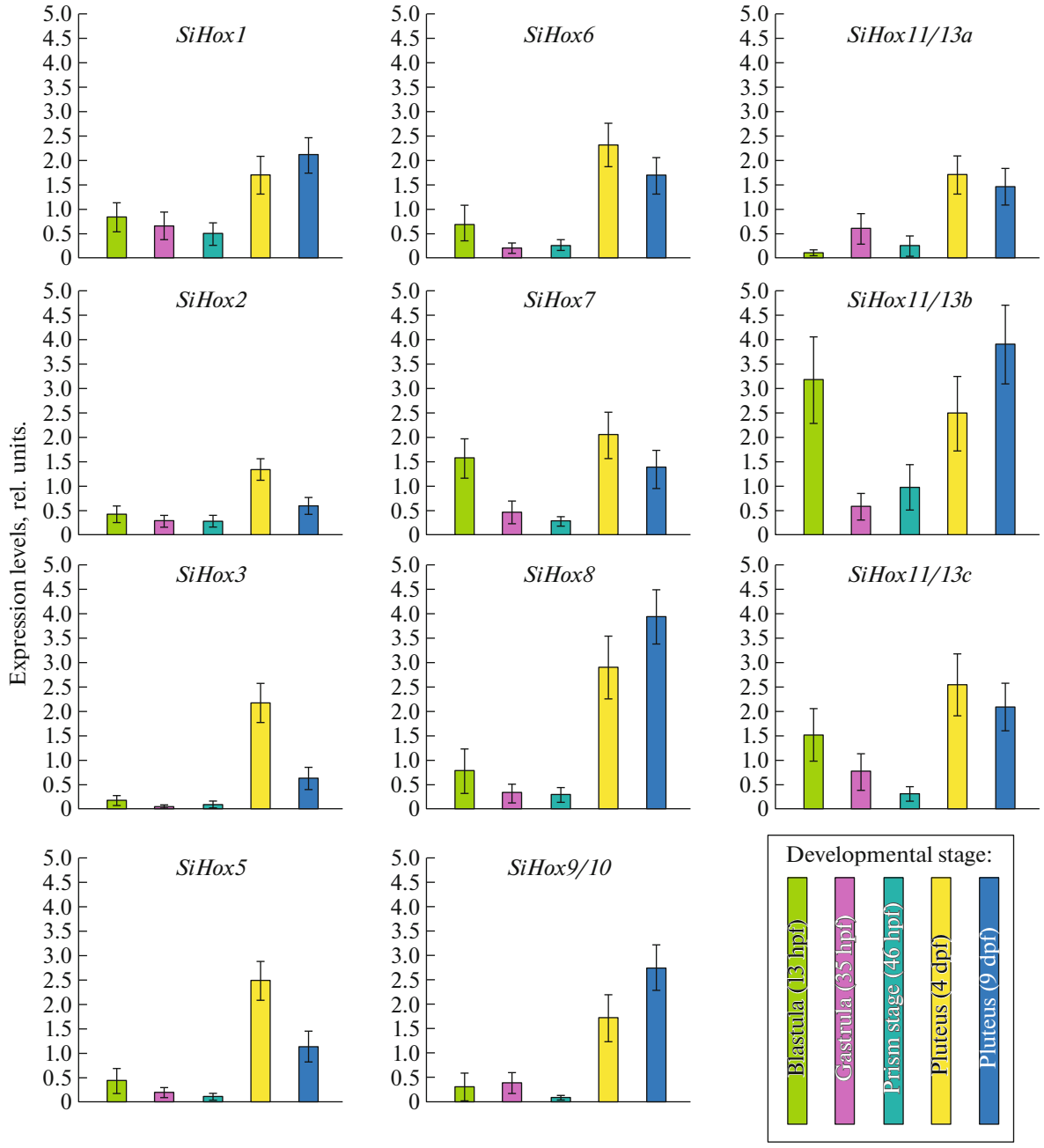

Fig. 2. Quantitative assessment of the expression levels of the *Hox* cluster genes in *S. intermedius* at early developmental stages (in relative units (rel. units)).

increase in expression of *SiHox1*, *8*, *9/10,* and *11/13b* and a slight decrease in expression of *SiHox5*, *6*, *7*, *11/13a,* and *11/13c*, although their expression levels were still higher than at the gastrula and prism stages. The *SiHox2* and *SiHox3* expression levels dropped below 1 rel. unit on 9 dpf (Fig. 2). According to the literature, an abrupt increase in expression of the *Hox* genes (*SpHox2*, *3*, *4/5*, *7*, *8*, *9/10*, *11/13a,* and *11/13b*) is similarly characteristic of *S. purpuratus* larvae at 14 and 21 days of age [10]. The majority of the *Hox* cluster genes are expressed to a substantial level in *P. japonica* plutei aged 1.5–4 days, with the exception of *PjHox6*, which is expressed to a minor level, and *PjHox11/13c*, which is not expressed at all after early gastrulation [11].

Our findings support the data that the earliest activation is characteristic of *Hox* genes of the central and posterior groups of the cluster (*Hox7*, *11/13b,* and *11/13c*), rather than of the anterior *Hox* gene group, in sea urchins. The same feature has been observed in the development of the sea cucumber *Apostichopus japonicus*; e.g., genes of the central and posterior groups of the *Hox* cluster (*AjHox7*, *8*, *11/13a,* and *11/13b*) are expressed at the blastula stage (12–20 hpf). The *AjHox1*, *5,* and *11/13c* genes are expressed in addition

to the above ones at the gastrula stage (24–34 hpf) [17]. Expression of the *Hox* genes at the blastula stage (20 hpf) has not been detected at all in the stalked crinoid *Metacrinus rotundus*; however, only *MrHox1*, *2*, *4*, *5*, *7*, *8*, *9/10,* and *11/13c* have been included in the study [18]. Only *MrHox7* is expressed in *M. rotundus* at the gastrula stage (40 hpf); *MrHox8* starts expression in addition to *Hox7* 2 days later (53 hpf); and *MrHox5*, *7*, *8,* and *9/10* are activated 4 dpf [18]. Thus, genes of the anterior group of the *Hox* cluster are silent in echinoderms at the earliest developmental stages, while only genes of the central and posterior groups are expressed, as demonstrated in all available studies of the temporal expression pattern of the *Hox* gene cluster.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. In accordance with Directive 2010/63/EU of September 22, 2010 on the protection of animals used for scientific purposes, Chapter 1, Paragraph 3, the requirements of bioethics do not apply to the subject of this study.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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