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Thermal induction of heat shock proteins Hsp70 and Hsp90 in tissues of the nemerteans *Lineus alborostratus* Takakura, 1898 and *Quasitetrastemma stimpsoni* (Chernyshev, 1992)

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ABSTRACT: Intertidal ribbon worms in the phylum Nemertea live in environments where temperature and salinity can vary widely depending on the tidal fluctuations. Heat shock proteins (Hsps) are expressed by cells in their physiological responses to these changing abiotic factors. To understand the role of Hsps, the presence of Hsp70 and Hsp90 was determined by western blotting in two nemertean species, *Lineus alborostratus* and *Quasitetrastemma stimpsoni* after 2- and 4-h thermal stress. Additionally, their tissue distributions of these Hsps were detected by immunohistochemistry. Both Hsps were detected in the two nemertean species. In *L. alborostratus*, Hsp70 and Hsp90 immunoreactivities were detected in the body wall, specifically in the epidermal and cutis cells, as well as in the epithelia of the stomach and proboscis; Hsp immunoreactivities in these cells and tissues appeared higher in the thermally-stressed nemerteans compared to the controls. For both the control and thermally-stressed *Q. stimpsoni*, Hsp70 and Hsp90 were localized in the epidermis, while the stomach epithelium and neurons of the lateral nerve cord showed low immunoreactivities in the thermally-stressed worms. In thermally-stressed *L. alborostratus*, the proboscis showed a marked increase of Hsp70 and Hsp90 immunoreactivity. Active immunoreactivities were observed in the supra-epidermal granules in both ribbon-worm species, especially *Q. stimpsoni*. Accumulation of Hsp70 and Hsp90 in the body wall and the gastric epithelium of nemerteans may indicate their importance during their physiological response to thermal stress.
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KEY WORDS: Hsp70, Hsp90, thermal stress, nemerteans, immunohistochemistry.

Термическая индукция белков теплового шока Hsp70 и Hsp90 у немертин *Lineus alborostratus* Takakura, 1898 и *Quasitetrastemma stimpsoni* (Chernyshev, 1992)

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РЕЗЮМЕ: Литоральные немертины живут в условиях, где температура и соленость может изменяться в широких пределах в зависимости от приливных колебаний. Экспрессия белков теплового шока (Hsp) является физиологической реакцией в ответ на эти изменяющиеся абиотические факторы среды. Для понимания их роли HSPs в адаптации методами вестерн-блоттинга и иммуногистохимии изучали наличие и распределение Hsp70 и Hsp90 у двух видов немертин, *Lineus alborostratus* и *Quasitetrastemma stimpsoni* после 2- и 4-часового теплового стресса. Hsps обнаружены у двух видов немертин. У *L. alborostratus* Hsp70 и Hsp90-иммунореактивность обнаружена в стенке тела в клетках эпидермиса и кутиса, а также в эпителии желудка и хобота, при температурном стрессе Hsp-иммунореактивность в этих клетках и тканях выше по сравнению с контрольной группой. *У Q. stimpsoni* в контроле и при тепловом стрессе Hsp70 и Hsp90 выявляются в эпидермисе, кроме того при стрессе низкая иммунореактивность выявляется в эпителии желудка и в нервных клетках боковых нервных стволов. У *L. alborostratus* при тепловом стрессе наблюдается увеличение Hsp70 и Hsp90 иммунореактивности в хоботе. Увеличение иммунореактивности эпидермального гранул выявляется в обоих видов немертин, особенно *Q. stimpsoni*. Накопление Hsp70 и Hsp90 в стенке тела и гастроэпидермисе немертин может указывать на их важную роль в физиологичесой устойчивости к термальному стрессу.

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КЛЮЧЕВЫЕ СЛОВА: Hsp70, Hsp90, тепловой стресс, немертины, иммуногистохимия.

Introduction

Elevated temperatures even a few degrees above optimal can cause living proteins of organisms to unfold, entangle, and aggregate non-specifically (Richter et al., 2010). Heat shock response of cells and tissues presumably is not affected directly by temperature but to the accumulation of these unfolded proteins (Richter *et al*., 2010). Arrigo (2000) reported that organisms respond to lethal effects of hyperthermia through protection of heat shock proteins (Hsps), a family of highly conserved proteins that are found in all organisms from prokaryotes to mammals (Hunt, Morimoto, 1985; Lindquist, 1986; Fast *et al*., 2002; Chen *et al*., 2015). Hsps are necessary for the normal functioning of the cell structures in response to environmental stresses (Lindquist, Craig, 1988; Sanders, 1993; Feder, Hofmann, 1999; Oyake *et al*., 2006).

The number categorization of each Hsp depends on the molecular weight (kDa), with each family containing individual protein isoforms. The more commonly recognized proteins are Hsp60, Hsp70, Hsp90, and Hsp100 (Parsell, Lindquist, 1993; Moseley, 1997; Borkan, Gullans, 2002), with Hsp70 and Hsp90 being the most ubiquitous of the Hsps.

Under normal conditions, Hsp70 contributes to the synthesis of new proteins in the cytosol and cell organelles (Mayer, Bukau, 2005). Hsp90, found in high concentrations in the cytosol, binds to native but not unfolded proteins (Richter *et al*., 2010). Under thermal stress Hsp70 and Hsp90 prevent aggregations of unfolded proteins (Richter *et al*., 2010) and

refolding of damaged proteins (Glover, Lindquist, 1998; Maloyan *et al*., 1999; Young *et al*., 2004). Furthermore, Hsp70 may refold aggregated proteins (Mayer, Bukau, 2005) while Hsp90 may be important in holding protein intact (Richter *et al*., 2010).

Hsp70 consists of three functional domains, ATP-dependent chaperones have a conserved N-terminal ATPase domain, a substrate binding domain, and a variable C-terminal region. Eukaryotic Hsp90 proteins have functionally conserved N-termini and C-termini connected by a middle region containing highly charged and hydrophobic regions with variable length (Pratt, Toft, 2003). Conserved sequences for Hsp70 and Hsp90 have been identified in human, mouse, rat, insects, nematodes, yeasts, plants, and virus (Hunt, Morimoto, 1985; Choi *et al*., 2008; Liang *et al*., 2013; Chen *et al*., 2015). The sequence identities ranged from 76.7 to 80.7% for Hsp70 and 73.8 to 77.6% for Hsp90 between human and nematodes (Chen *et al*., 2015).

In invertebrates, Hsp is induced by various environmental factors, including osmotic (Tirard *et al*., 1997; Okazaki *et al*., 2006) and oxidative stress (Mager *et al*.á 2000), hypo- and hyperthermia, anoxia/hypoxia (Chang *et al*., 2000), and heavy metal pollution (Piano *et al*., 2004; Pruski, Dixon, 2007; Lilja *et al*., 2008). The physiological responses of these invertebrates allow them to be considered as biomarkers of stress (Ackerman *et al*., 2000; Bierkens, 2000).

Temperature is one of the major factors influencing the functional state of marine invertebrates on all levels of biological organization, including molecular (Hochachka, Somero, 2002), and can lead to the damage of proteins (Hamdoun *et al*., 2003). Two distinct Hsp families, Hsp70 and Hsp90, are currently recognized to have a central role in response against thermal stress; their presence under thermal stress has been found in many organs and tissues of invertebrates from various taxonomic groups (Sanders *et al*., 1991; Hofmann, Somero, 1996; Clegg *et al*., 1998; Tomanek, Somero, 1999; Spees *et al*., 2002; Boutet *et al*., 2003; Hamdoun *et al*., 2003). The cytoprotective function of Hsp allows the nematode *Caenorhabditis elegans* to be highly resistant to a variety of environmental stress stimuli (Prahlad *et al*., 2008).

In the nemertean *Paranemertes peregrina* Hsp70 and Hsp90 were easily induced by heat and osmotic stresses, resulting in expression level detected by immunoblot (Okazaki *et al*., 2001, 2006). However, very little is known about both Hsp70 and Hsp90 in response to thermal stress in other species of nemerteans. In Vostok Bay in the Sea of Japan, two different taxonomic orders of nemerteans, *Lineus alborostratus* Takakura, 1898 (Heteronemertea: Lineidae) and *Quasitetrastemma stimpsoni* (Chernyshev, 1992) (Hoplonemertea: Tetrastemmatidae) co-exist. *L. alborostratus* has been found in habitats varying in temperatures from –2°C in winter to 24°C in summer; whereas *Q. stimpsoni* has adapted to higher temperatures (Chernyshev, 2014). Chernyshev (2014) reported two different reproductive temperatures: 10–15°C for *L. alborostratus* and 20–22°C for *Q. stimpsoni.* Thus, we hypothesize that the two species would show differences in Hsp70 and Hsp90 responses in reflecting their varying ecological thermal environments. Additionally, distribution of these Hsps in nemertean tissues has not been reported. To address this lack of information, immunohistochemistry was used to determine the distribution of Hsp70 and Hsp90 in the tissues of *L. alborostratus* and *Q. stimpsoni* under thermal stress. Identification of specific tissues involved in stress response may be helpful in understanding a role of Hsp in the protection of ribbon worms in their fluctuating thermal environments.

Materials and Methods

Experimental materials

Adult specimens of *Lineus alborostratus* and *Quasitetrastemma stimpsoni* were collected in intertidal zone of Vostok Bay (42°52′33″N, 132°44′35″E) in the Sea of Japan in June 2013. The ribbon worms were acclimated in an aerated seawater aquarium (5°C at 33‰) for three

weeks. Good ethical procedures have been followed in the care, housing, and use of the animals in our study in accordance to the European Communities Council Directive of 24 November 1986 (86/609/EEC). In the experiment, 36 nemerteans were divided into three treatments: a control group (6 specimens of each species) was maintained at a temperature of 5°C and two experimental treatments (6 specimens of each species) were each exposed to thermal stress (22°C) for 2 h and 4 h. This temperature was selected after experimental observations that determined the lethal temperature to be 26–27°C. The maximum of 4-hr duration of the thermal-stress experiment was selected based on preliminary testing that revealed that the rapid rise from 5°C to 22°C was sub-lethal after 4–5 h for both nemertean species and lethal after 6 h for *Q. stimpsoni* and 7–8 h for *L. alborostratus*.

Western immunoblotting

For western blotting, three specimens of each species from each treatment were homogenized (1:5) in the buffer containing 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation (15,000 g at 4°C, 20 min), the supernatant was separated, and the total protein concentration was determined by the Lowry method (Lowry *et al*., 1951). Then, 40 μg of proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) at 150 V for 1 h. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Sigma-Aldrich, USA) and left overnight in 0.01 mM TBS (0.01 mM Tris–HCl buffer, pH 8.0, 0.15 mol^{-1} NaCl) containing 4% bovine serum albumin (BSA). The membranes were rinsed in distilled water and incubated with mouse monoclonal antibodies against Hsp70 (Abcam, UK; Cat. #2787) or Hsp90 (Abcam, UK; Cat. #1429) diluted 1:1000 in TBS containing 1% BSA for 3 h at 4°C. After washing in TBS, the membranes were incubated with horseradish-peroxidase conjugated secondary antibodies (dilution 1:200, Vector Labs, USA; Cat. #PK-6200) for 1 h at room temperature. Peroxidase reaction was visualized using a VIP Substrate Kit (Vector Labs, USA). The membranes were washed and scanned. Molecular mass determinations were based on prestained molecular mass markers (GeneDirex, Inc., USA).

Immunohistochemical detection

For immunohistochemical detection of Hsp70 and Hsp90, the remaining 18 nemerteans were fixed whole in 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) (pH 7.2) for 2 h at 4°C. After washing in PBS, they were placed for 1 day in a cold solution (30%) of sucrose in 0.1 M PBS. Hsp70 and Hsp90 were detected using the methods developed by Chernyshev & Kotsyuba (2014). Thick sections (~30–35 μm) were cut on a freezing microtome and mounted on polylysine-coated slides. After inhibition of endogenous peroxidase in 1% H_2O_2 and suppression of non-specific binding of antibodies in 1% normal goat serum, the sections were then incubated with mouse monoclonal antibodies against Hsp70 or Hsp90 (1:500; Abcam, UK) for 18 h at 4°C, which shows cross-reactivity with mouse, rat, rabbit, cow, human, *Saccharomyces cerevisiae*, bird, *Drosophila melanogaster*, fish, and amphibians (Monribot-Villanueva et al., 2013; Zhang *et al*., 2015; Mario *et al*., 2016). The sections were washed in three changes of 0.1 M PBS (pH 7.2) and incubated with biotinylated secondary antibodies against mouse immunoglobulin (Ig) (1:200) (Vector Labs, USA; Cat. #PK-6200) for 2 h. After washing, all of the sections were then incubated with anavidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Labs, USA; Cat. #PK-6200) for 1 h at 22°C in the dark and then washed thrice in PBS. The reaction products were visualized using a substrate (VIP Substrate Kit, Vector Labs, USA; Cat. #SK-4600). Afterwards, the sections were washed in three changes of PBS, dehydrated through an ethanol series of increasing concentrations, and mounted in Canadian balsam. The corresponding negative control sections were prepared by

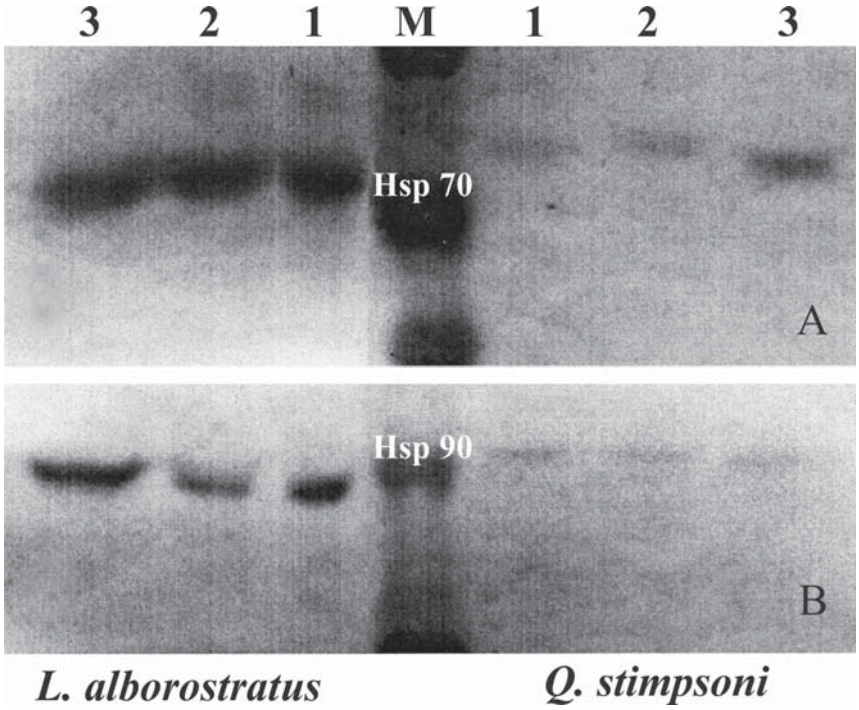


Fig. 1. Western immunoblots of Hsp70 and Hsp90 in *Lineus alborostratus* and *Quasitetrastemma stimpsoni* in the (control) 0-, 2- and 4-h thermal stress. A — Hsp70; B — Hsp90.
Abbreviations: Lane M — pre-stained molecular mass standards, kDa; Lane 1 — control group 0-h; Lane 2 — 2-h thermal stress; Lane 3 — 4-h thermal stress.
Рис. 1. Вестерн-иммуноблоттинг Hsp70 и Hsp90 у *Lineus alborostratus* и *Quasitetrastemma stimpsoni* (контроль) 0-, 2- и 4-часовой тепловой стресс. A — Hsp70; B — Hsp90.
Обозначения: дорожка M — преокрашенные стандарты молекулярной массы, кД; дорожка 1 — контрольная группа 0 ч; дорожка 2 — 2-часовой тепловой стресс; дорожка 3 — 4-часовой тепловой стресс.

omitting the primary antibody (Huang *et al*., 2005; Bao *et al*., 2008). The immunohistochemically-stained sections were photographed on an Axiovert 200 M inverted microscope (Zeiss, Göttingen, Germany) with a digital microscopy camera (Zeiss AxioCam HRC, Göttingen, Germany).

The number of Hsp70- and Hsp90-positive cells in specific nemertean tissues was estimated using an automatic image analysis system Allegro-MC (Afanasyev et al., 2002). The system included an ERMA-2 microscope (Japan) equipped with a CREATIVE video camera (Japan) and IBM-compatible computer with a video capture card ATI-128 pro (32 MB, 1280 × 1024 pixel). In each section, Hsp70 and Hsp90 immunoreactive cells were counted in five randomly selected fields of vision using VideoTest 5.0 software (VideoTest Software, St. Petersburg, Russia) to obtain a percentage (number of immunoreactive cells/total cells counted). For each tissue, Hsp70 and Hsp90 immunoreactive cells were then counted from 10 histological sections.

Statistical analysis

All percentages were converted by taking their square roots and then calculating their arcsin values. These transformed values were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test (Vassarstats Program) to determine significant differences ($P < 0.05$) among the control, 2-h, and 4-h treatments (Sokal, Rolhf, 1994).

Results

Western immunoblotting

The immunoblots revealed apparent differences in the concentrations of Hsp70 and Hsp90 between the two ribbon worm species from the control and thermally-stressed treatments. With equal starting protein loadings (40 μg) in the control for both species, the bands of both Hsp70 and Hsp90 were markedly present in *L. alborostratus* but barely noticeable in *Q. stimpsoni* (Fig. 1A, B). After 2-h and 4-h thermal stress for *L. alborostratus,* Hsp70 appeared to show a weak increase compared to the controls while for *Q. stimpsoni,* Hsp70 showed a marked increase after 4-h thermal stress. In *L. alborostratus* Hsp90 bands were moderately visible at 2-h thermal stress but after 4-h stress its concentrations appeared to increase dramatically. In *Q. stimpsoni* the amount of Hsp appeared to be quite low as the bands were barely discernible.

Control Hsp70 immunohistochemistry

Immunohistochemical analysis revealed Hsp70 and Hsp90 in both nemertean species; however, their tissue distributions in the control and thermally-stressed worms differed (Figs. 2–7). For the control *Q. stimpsoni*, immunoreactivity was generally detected in the epidermal cells of the anterior of worm, particularly the precerebral region but was rarely found in the posterior region. In *L. alborostratus* Hsp70 immunoreactivity was detected in the epidermal cells of all regions. Hsp70 immunoreactivity was not observed around the stomach cells of *L. alborostratus* (Fig. 2A) and *Q. stimpsoni* (Fig. 2B). The negative controls did not bind non-specifically to any cell or tissue of *L. alborostratus* (Fig. 2A) and *Q. stimpsoni* (Fig. 2B). However, for the two nemertean species, positive Hsp70 immunoreactivities were detected in the epidermal cells (Fig. 2E, F) and supra-epidermal granules (i.e. compact particles on the epidermal surface) and localized separately or in small clusters (Fig. 2C, D). In *L. alborostratus*, Hsp70 immunoreactivities were also observed in the stomach epithelia but not around the stomach walls (Fig. 2G) and in the separate cells of the proboscis epithelium (Fig. 2H).

Thermal stress Hsp70 immunohistochemistry

After 2-h thermal stress in *Q. stimpsoni*, Hsp70 immunoreactivity appeared to increase

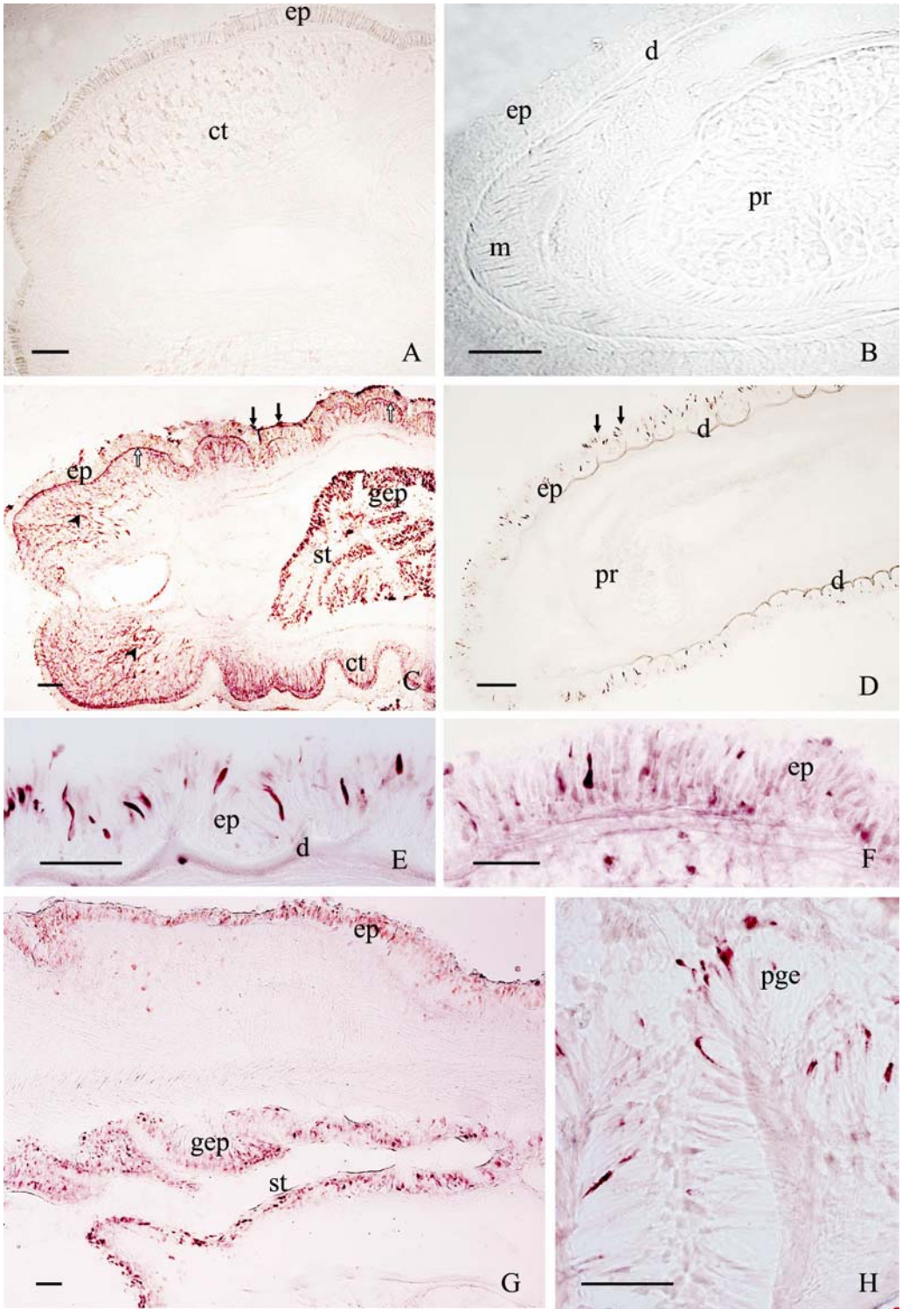
Fig. 2. Distribution of Hsp70 in *Lineus alborostratus* and *Quasitetrastemma stimpsoni*. A — *L. alborostratus*, negative control section showing no Hsp70-immunoreactivity; B — *Q. stimpsoni*, negative control section showing no Hsp70-immunoreactivity; C — *L. alborostratus*, positive control section showing the presence of Hsp70 immunoreactivity in the stomach region of *L. alborostratus*; D, E — *Q. stimpsoni*, positive control section showing the presence of Hsp70 immunoreactivity (white arrows) in the basal epidermal layer of *Q. stimpsoni*; F — Hsp70-immunoreactivity in the epidermis of *L. alborostratus*; G — stomach of *L. alborostratus*; H — proboscis epithelium of *L. alborostratus* after 0-h thermal stress. Black arrows indicate immunoreactive supra-epidermal granules, arrowheads show cephalic glands.

Abbreviations: ct — cutis; d — dermis; ep — epidermis; gep — gastral epithelium; m — body musculature; pge — proboscis gland epithelium; pr — proboscis; st — stomach. Scale bar: A — 50 μm; B–D — 100 μm; E–H — 50 μm.

Рис. 2. Распределение Hsp70 у *Lineus alborostratus* and *Quasitetrastemma stimpsoni*. A — *L. alborostratus*, негативный контроль показывает отсутствие Hsp70-иммунореактивности в области желудка; B — *Q. stimpsoni*, негативный контроль показывает отсутствие Hsp70-иммунореактивности в области желудка; C — *L. alborostratus*, позитивный контроль показывает Hsp70-иммунореактивность в области желудка; D, E — *Q. stimpsoni*, положительный контроль показывает наличие Hsp70-иммунореактивности (белые стрелки) в базальной части эпидермиса; F — распределение Hsp70-иммунореактивности в контроле в эпидермисе у *L. alborostratus*; G — желудок *L. alborostratus*; H — эпителий хобота *L. alborostratus*, контрольная группа 0 ч.

Черные стрелки указывают на иммунореактивные гранулы на поверхности эпидермиса, наконечники стрелок указывают на головные железы.

Обозначения: ct — кутис; d — дермис; ep — эпидермис; gep — гастроэпителий; m — мускулатура стенки тела; pge — эпителий хобота; st — желудок. Масштаб: A Ï 50 мкм; B–D — 100 мкм; E–H — 50 мкм.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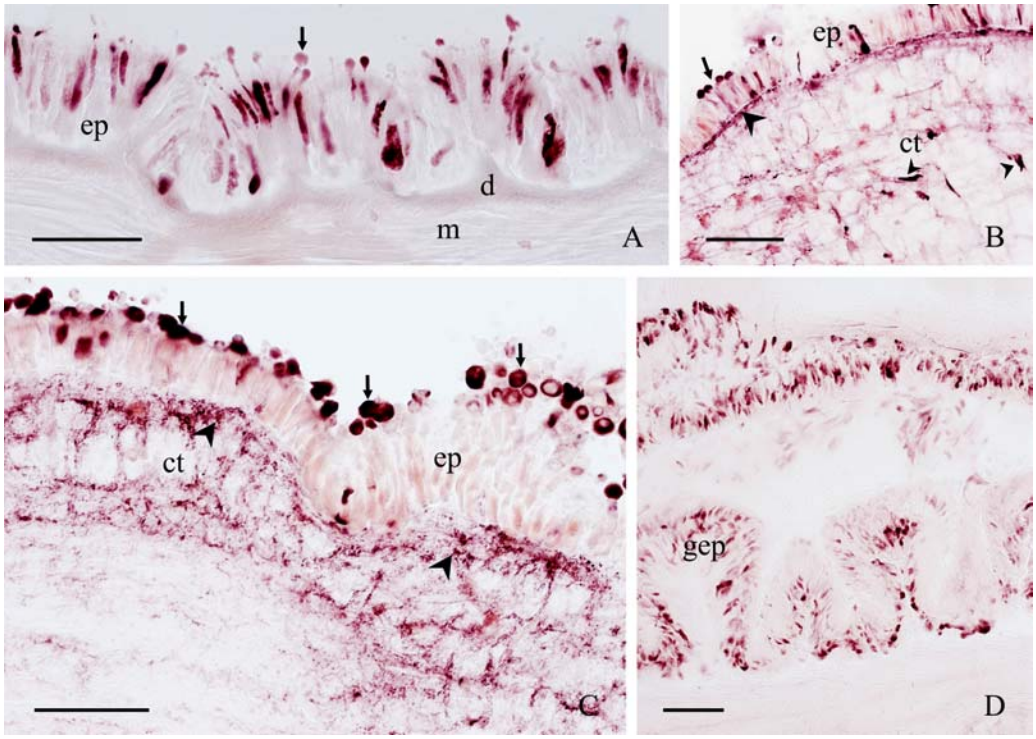

Fig. 3. Distribution of Hsp70 immunoreactivity in *Lineus alborostratus* and *Quasitetrastemma stimpsoni* in the body wall and digestive system after 2-h thermal stress. A — *Q. stimpsoni*; B–D — *L. alborostratus*; A–C — the body wall; D — digestive system. Arrows indicate immunoreactive supra-epidermal granules, larger arrowheads show subepithelial glands, smaller arrowheads indicate subepithelial layer ("plexus").
Abbreviations: ct — cutis; d — dermis; ep — epidermis; gep — gastral epithelium; m — body musculature. Scale bars: 50 μm.

Рис. 3. Распределение Hsp70-иммунореактивности у *Quasitetrastemma stimpsoni* и *Lineus alborostratus* в стенке тела и пищеварительной системе после 2-часового теплового стресса. A — *Q. stimpsoni*; B–D — *L. alborostratus*; A–C — стенка тела; D — пищеварительная система.
Стрелки указывают на иммунореактивные гранулы, большие наконечники стрелок указывают на субэпителиальные железы, маленькие наконечники стрелок указывают на субэпидермальный слой.
Обозначения: ct — кутис; d — дермис; ep — эпидермис; gep — гастроэпителий; m — мускулатура стенки тела. Масштаб: 50 мкм.

substantially in some areas of the epidermis, specifically, the release of Hsp70-immunoreactive granules were observed on the surface of the epidermal cells (Fig. 3A). In *L. alborostratus* Hsp70 immunoreactivity of separate cells in the epidermis appeared to show little increase (Fig. 3B). However, intensive reaction was observed in the small non-glandular cells (4–7 μm in diameter) of the thin subepidermal layer of the extracellular matrix and larger glandular cells (12–20 μm long), which were localized deep in the cutis in the anterior body part, predominantly in the precerebral region (Fig. 3B, C). Moreover, the aggregations of Hsp70 immunoreactive granules (6–10 μm) appeared on the surface of the epidermis; their number and intensity of staining varied in different parts of the body (Fig. 3B, C). In the digestive system of *L. alborostratus*, Hsp70 immunoreactivity in the epithelial cells of the stomach and intestine showed an increase compared to the control (Fig. 3D).

After 4-h thermal stress in *Q. stimpsoni,* Hsp70 immunoreactivity increased in the distal

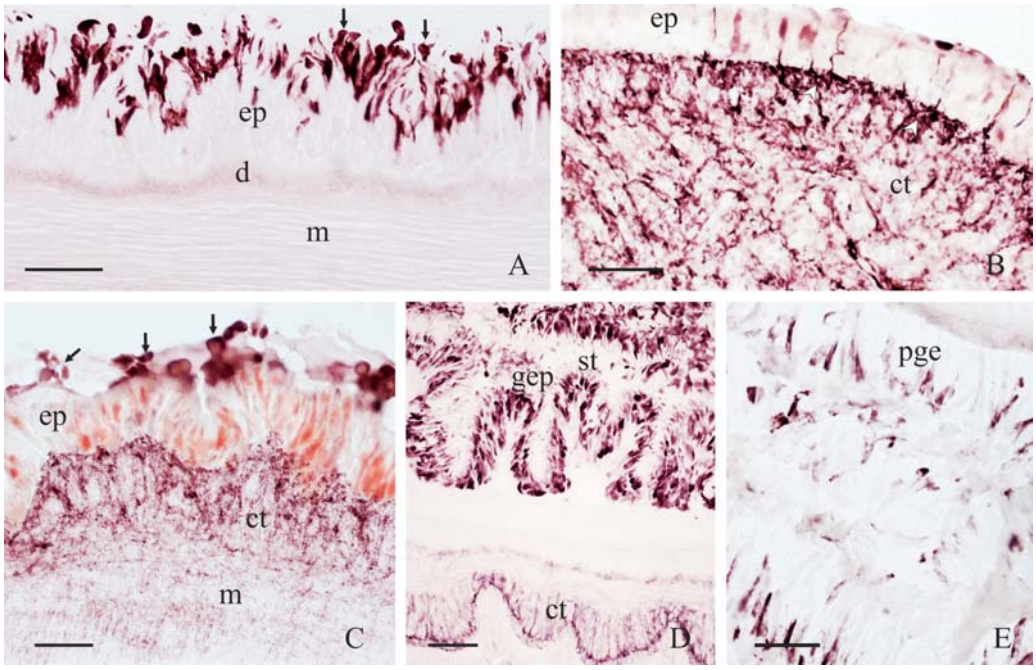

Fig. 4. Distribution of Hsp70 immunoreactivity in *Lineus alborostratus* and *Quasitetrastemma stimpsoni* in the body wall, digestive system, and proboscis after 4-h thermal stress. A — *Q. stimpsoni*; B–E — *L. alborostratus*; A–C — in the body wall; D — digestive system; E — proboscis. Arrows indicate immunoreactive supra-epidermal granules, arrowheads show subepithelial layer.

Abbreviations: ct — cutis; d — dermis; ep — epidermis; gep — gastral epithelium; m — body musculature; st — stomach; pge — proboscis gland epithelium. Scale bars: 50 μm.

Рис. 4. Распределение Hsp70-иммунореактивности у *Quasitetrastemma stimpsoni* и *Lineus alborostratus* в стенке тела, пищеварительной системе и хоботе после 4-часового теплового стресса. A — *Q. stimpsoni*, B–E — *L. alborostratus*, A–C — стенка тела; D — пищеварительная система; E — хобот. Стрелки указывают на иммунореактивные супраэпидермальные гранулы, наконечники стрелок указывают на субэпителиальный слой.

Обозначения: ct — кутис; d — дерма; ep — эпидермис; gep — гастроэпителий; m — мускулатура стенки тела; pge — эпителий хобота; st — желудок. Масштаб: 50 мкм.

part of epidermis (Fig. 4A). In *L. alborostratus*, Hsp70 immunoreactivity was observed in the isolated epidermal cells (Fig. 4B, C). Hsp70 immunoreactive material was evenly distributed over the apical surface of the epidermis (Fig. 4C) as aggregations of Hsp70 immunoreactive granules were only found in some areas. Also in the cutis, the immunoreactivity increased; with the highest amount observed in the anterior part of the body, mainly in the precerebral region (Fig. 4B). Hsp70 immunoreactivity also appeared in the body wall musculature (Fig. 4C). In the digestive system, the number and intensity of staining of Hsp70 immunoreactive cells in the stomach epithelium increased (Fig. 4D); extracellular distribution of immunoreactive material was observed in the lumen of the stomach. Hsp70 immunoreactivity was detected mainly in the proximal part of the glandular epithelium of the proboscis (Fig. 4E).

Control Hsp90 immunohistochemistry

For the control *Q. stimpsoni*, Hsp90 was revealed in the epidermal cells and secretory granules on the epidermal surface (Fig. 5A). In *L. alborostratus,* Hsp90 was detected in the

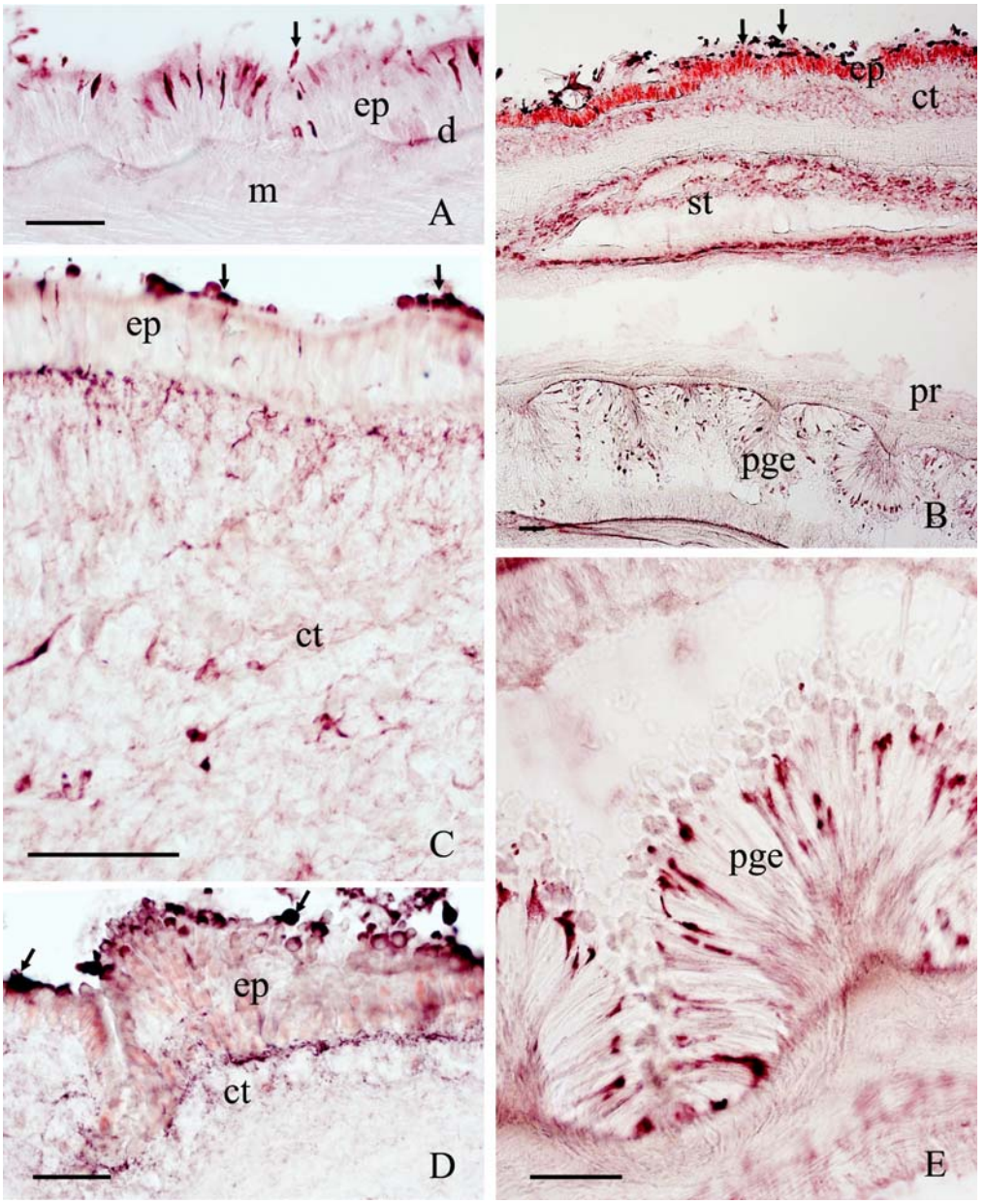

Fig. 5. Control Hsp90 immunoreactivity in *Quasitetrastemma stimpsoni* and *Lineus alborostratus* in the body wall, stomach, and proboscis. A — *Q. stimpsoni*; B–E — *L. alborostratus*; A, C, D — body wall; B — body wall, stomach and proboscis; E — proboscis. Arrows indicate immunoreactive supra-epidermal granules.
Abbreviations: ct — cutis; d — dermis; ep — epidermis; m — body musculature; pge — proboscis gland epithelium; st — stomach. Scale bars: 50 μm.

Рис. 5. Hsp90-иммунореактивность в стенке тела, желудке и хоботе у *Quasitetrastemma. stimpsoni* и *Lineus alborostratus* в контроле. A — *Q. stimpsoni*; B–E — *L. alborostratus*; A, C, D — стенка тела; B — стенка тела, желудок и хобот; E — хобот.
Стрелки указывают на иммунореактивные супраэпидермальные гранулы.
Обозначения: ct — кутис; d — дерма; ep — эпидермис; m — мускулатура стенки тела; pge — эпителий хобота; st — желудок. Масштаб: 50 мкм.

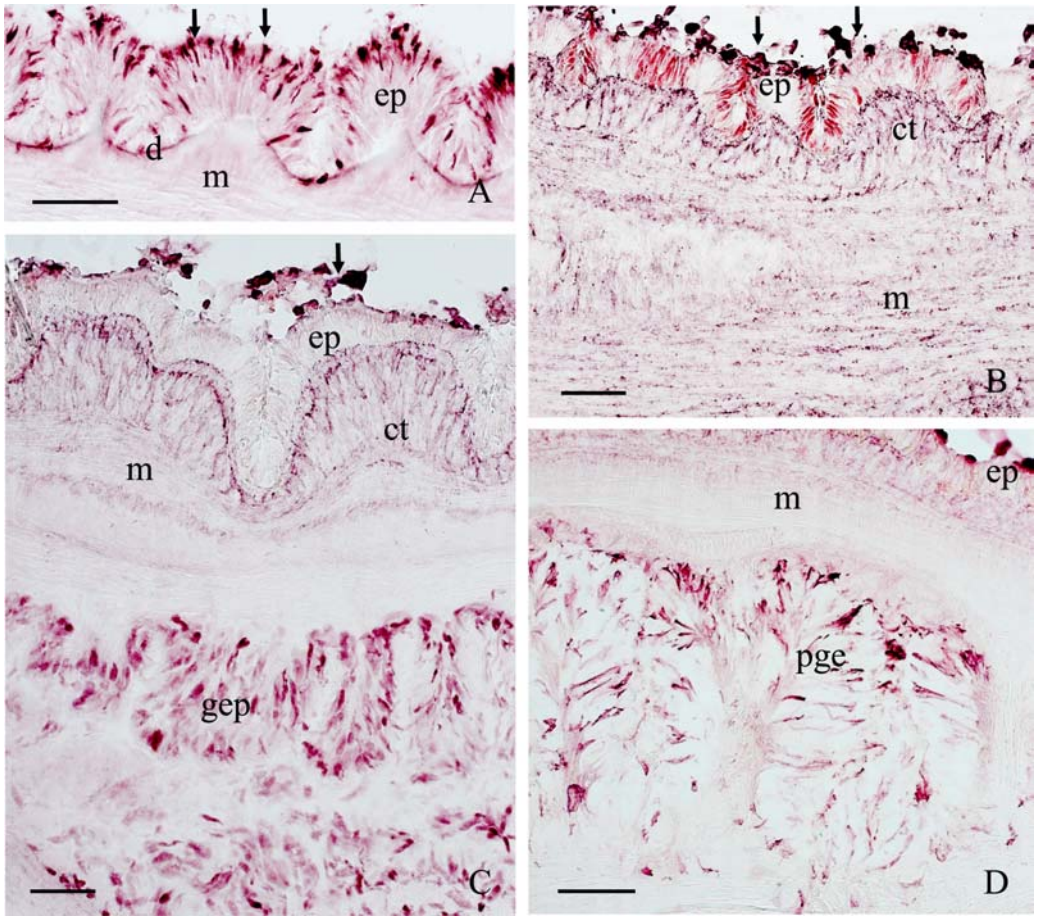

Fig. 6. Distribution of Hsp90 in *Lineus alborostratus* and *Quasitetrastemma stimpsoni* after 2-h thermal stress. A — *Q. stimpsoni*; B–D — *L. alborostratus*. A–B — body wall; C — body wall and stomach; D — proboscis. Arrows indicate immunoreactive supra-epidermal granules. Abbreviations: ct — cutis; d — dermis; ep — epidermis; m — body musculature; gep — gastral epithelium; pge — proboscis gland epithelium. Scale bars: 50 μm.

Рис. 6. Распределение Hsp90 у *Quasitetrastemma stimpsoni* и *Lineus alborostratus* после 2-часового теплового стресса. A — *Q. stimpsoni*; B–D — *L. alborostratus*. A–B — стенка тела; C — стенка тела и желудок; D — хобот.

Стрелки указывают на иммунореактивные супраэпидермальные гранулы. Обозначения: ct — кутис; d — дерма; ep — эпидермис; gep — гастроэпителий, m — мускулатура стенки тела; pge — эпителии хобота; st — желудок. Масштаб: 50 мкм.

body wall, stomach, and proboscis (Fig. 5B). In the body wall, Hsp90 immunoreactivity was revealed in the cutis (Fig.5C). Besides, granules of 5–10 μm were intensively marked over the entire epidermal surface (Fig. 5B–D). In the digestive system, Hsp90 immunoreactive cells were revealed in the stomach epithelium (Fig. 5B). In the proboscis, Hsp90 immunoreactivity was detected in the glandular epithelium (Fig. 5B, E).

Thermal stress Hsp90 immunohistochemistry

In 2-h thermally-stressed *Q. stimpsoni*, the number and intensity of staining of Hsp90 immunoreactive cells increased in the body wall, mainly in its distal part (Fig. 6A). In *L. alborostratus* a slight increase in the staining intensity of cells in the cutis and the subepidermal layer was observed (Fig. 6B). Besides, numerous

Hsp90-immunoreactive granules were detected on the epidermal surfaces. In the digestive system, the number of Hsp90 immunoreactive cells increased in the glandular cells with the basal part of the epithelium stained most intensively (Fig. 6C). Pronounced Hsp90 immunoreactivity was also observed in the glandular epithelium of the proboscis (Fig. 6D).

In the 4-h thermally-stressed *Q. stimpsoni*, Hsp90 immunoreactivity was revealed in the distal part of the epidermis (Fig. 7A). Hsp90 immunoreactivity appeared in single neurons of the lateral nerve cords (Fig. 7B), in cells of the stomach (Fig. 7C), and in the proboscis (Fig. 7D). Low immunoreactivities were detected in the proboscis, stomach, and lateral nerve cord. In *L. alborostratus*, a number of Hsp90-immunoreactive cells increased in the cutis (Fig. 7E). In the digestive system, Hsp90 immunoreactivity was visibly present in the gastric epithelium and in the lumen of the stomach (Fig. 7F). Additionally, faint Hsp90 immunoreactivities were observed at the boundary of the lateral nerve cords and outer longitudinal musculature (Fig. 7E).

Statistical analysis

The results of the statistical analysis are tabulated in Table 1. The epidermis showed marked differences in the Hsp immunoreactivities between the two ribbon-worm species (Fig. 8). In *Q. stimpsoni*, Hsp immunoreactivities were revealed locally within the epidermis (47–69% for Hsp70 and 38–55% for Hsp90), resulting in very highly significant differences ($P<0.001$) among the treatments (Fig. 8A). However, *L. alborostratus* showed no significant differences ($P=0.09$) in immunoreactivities of Hsp70 (19–20%) and Hsp90 (10–15%) among three treatments (Fig. 8D). For both species, the Hsp70 and Hsp90 immunoreactivities in the controls ranged from 11–20%. Interestingly, Hsp immunoreactivity of the supra-epidermal granules were observed to increase significantly ($P<0.0001$) at 2-hr in both the anterior and mid to posterior halves of *Q. stimpsoni* (Fig. 8B, C) and in *L. alborostratus* (Fig. 8E). In other tissues of *L. alborostratus*, significant differences ($P<0.0001$) in immunoreactivities were found (Fig. 9). For the sub-epithelial glands (Fig. 9A), a high Hsp70 immunoreactivity (53%) was detected in the 4-hr treatment. Similarly, in both the 2-h and 4-hr treatments, Hsp90 immunoreactivities were high (55%). The cutis cells showed high immunoreactivities, 63 and 39%, at 4-h treatments for Hsp70 and Hsp90, respectively (Fig. 9B). The most dramatic changes were detected in the stomach for 2-hr to 4-hr thermal stress; Hsp70 and Hsp90 immunoreactivities increased from 44 to 93% and 39 to 83%, respectively (Fig. 9C). High immunoreactivities, 36% for Hsp70 in the proboscis, were detected at 4-h treatment; whereas, for Hsp90, immunoreactivities of 24 and 26% did not differ between the 2-h and 4-hr treatments (Fig. 9D). Since no Hsp70 and Hsp90 immunoreactivities were detected in the lateral nerve cord of *L. alborostratus* and only Hsp90 at the 4-h treatment, statistical analysis was not performed for this tissue.

Discussion

This study showed the presence of Hsp70 and Hsp90 immunoreactivities in the tissues of intact *L. alborostratus* and *Q. stimpsoni*. Considerable differences in immunoreactivities of the two Hsps were observed between the two ribbon-worm species. Detection of Hsp70 and Hsp90 in both *L. alborostratus* and *Q. stimpsoni* confirms the previous report of these two Hsps in thermally stressed *Paranemertes peregrina* (Okazaki *et al*., 2001). Although the immunoblots showed very little Hsp70 and Hsp90 concentrations for *Q. stimpsoni*, the immunohistochemical analysis revealed the epidermis as its most sensitive tissue when compared to that of *L. alborostratus*. Other tissues of *Q. stimpsoni* showed very little or no Hsp immunoreactivities, while those other tissues of *L. alborostratus* were quite immunoreactive to the both Hsps.

Evgen'ev *et al*. (2007) reported varying Hsp concentrations in thermally stressed invertebrates depend on a number of factors, the species of animal, the type of cell and tissue, and the

Table 1. Results of the one way ANOVA statistical analysis of the percentages of Hsp70 and Hsp90 immunoreactivities in the different tissues and the supra-epidermal granules of *Lineus alborostratus* and *Quasitetrastemma stimpsoni*.

Таблица 1. Результаты однофакторного статистического анализа (ANOVA) процентных значений иммунореактивности Hsp70 и Hsp90 в различных тканях и супраэпидермальных гранулах *Lineus alborostratus* and *Quasitetrastemma stimpsoni*.

	P	F value	df	n	Tukey HSD	
***L. alborostratus* Hsp70**						
epidermis	0.0900	2.6	2	30	ns	
subepithelial glands	0.0001	455.6	2	30	4.9 (P<0.01)	
cutis	0.0001	4184.9	2	30	4.8 (P<0.01)	
stomach	0.0001	246.1	2	30	6.8 (P<0.01)	
proboscis	0.0001	48.8	2	15	7.6 (P<0.01)	
granules	0.0001	95.9	2	12	2.8 (P<0.01)	
***L. alborostratus* Hsp90**						
epidermis	0.1100	2.5	2	30	ns	
subepithelial glands	0.0001	183.3	2	30	6.1 (P<0.01)	ns: 2-h vs 4-h
cutis	0.0001	13.2	2	30	4.5 (P<0.01)	ns: Control vs 2-h
stomach	0.0001	228.8	2	30	6.3 (P<0.01)	
proboscis	0.0006	14.7	2	15	9.6 (P<0.01)	ns: 2-h vs 4-h
granules	0.7426	0.3*	8	10	ns	
***Q. stimpsoni* Hsp70**						
epidermis	0.0001	223.3	2	30	4.9 (P<0.01)	
supra-epidermal granules	0.0001	71.6	2	30	2.7 (P<0.01)	ns: 2-h vs 4-h
***Q. stimpsoni* Hsp90**						
epidermis	0.0001	73.4	2	30	5.6 (P<0.01)	
supra-epidermal granules	0.0079	6.1	2	25	4.0 (P<0.01)	ns: Control vs 2-h ns: Control vs 4-h

* t-value from t-test since 4-hr showed no but completely-fused granules.

temperature at which the cells (tissues, animals) were acclimatized before stress. In some invertebrate species, a high correlation between the tissue level of Hsp70 and their habitat temperatures has been found (Tomanek, Somero, 1999, 2000; Sørensen *et al*., 2005). Thus high Hsp concentrations in the control and thermally stressed *L. alborostratus* could be correlated with the ecological conditions of its habitat. This nemertean species lives predominantly in the intertidal zone of the Sea of Japan, where water temperatures can fluctuate greatly, e.g. from –2°C in winter to +24°C in summer (Chernyshev, 2014). Moreover, in hyperthermia experiments with fruit fly *Drosophila*, higher Hsp70 concentrations were found in a population from a cold rather than from a warm climatic zone (Sørensen *et al*., 2001, 2005).

This study found accumulation of Hsp70 in the body wall, stomach, and proboscis after 2-h

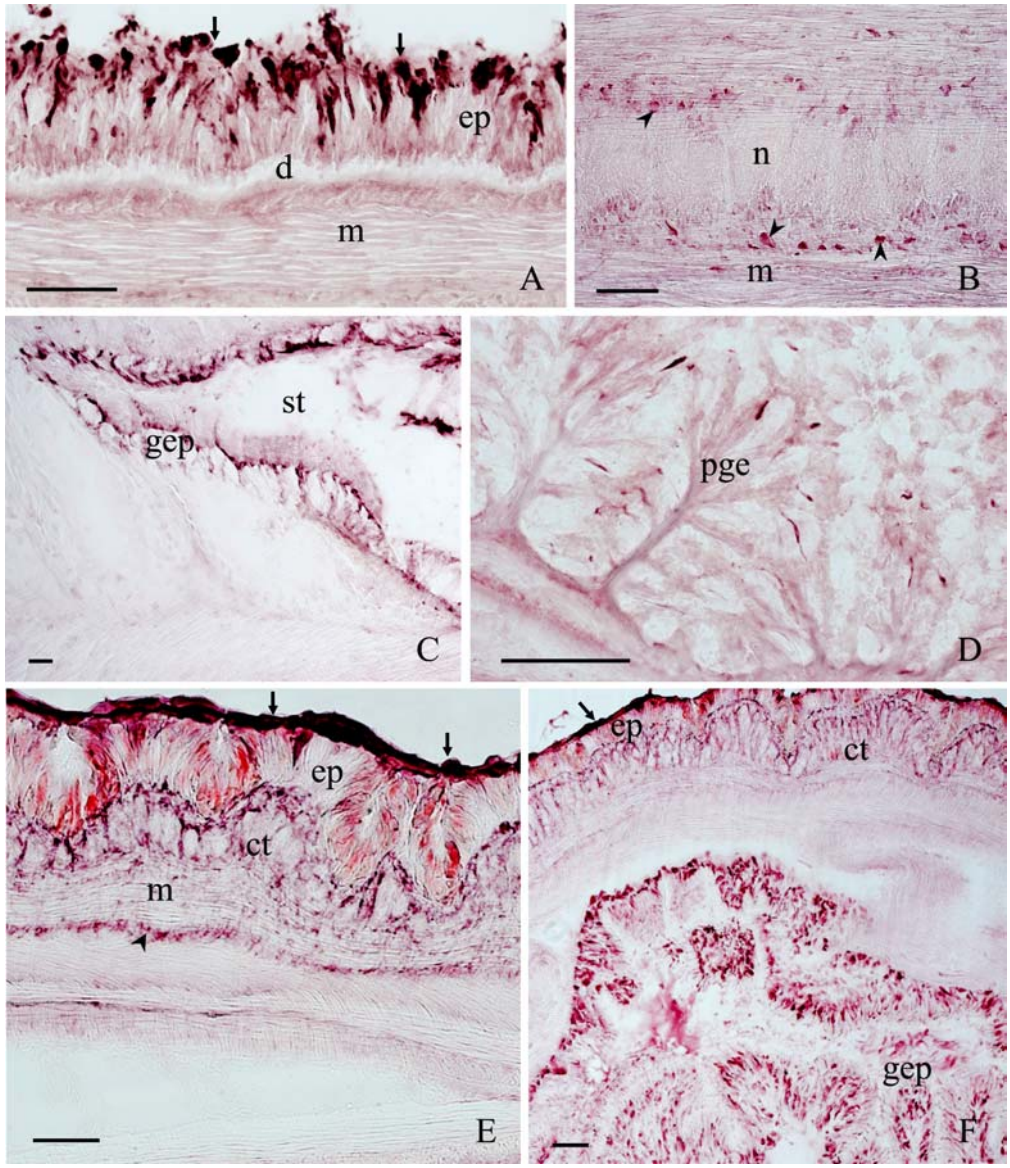

Fig. 7. Distribution of Hsp90 in *Quasitetrastemma stimpsoni* and *Lineus alborostratus* after 4-h thermal stress. A–D — *Q. stimpsoni*; E, F — *L. alborostratus*. A, E, F — body wall; B — lateral nerve; C, F — stomach; D — proboscis.

Arrows indicate immunoreactive supra-epidermal granules, arrowheads show lateral nerve and neurones.

Abbreviations: ct — cutis; d — dermis; ep — epidermis; gep — gastral epithelium; ln — lateral nerve; m — body musculature; pge — proboscis gland epithelium; st — stomach. Scale bars: 50 μm.

Рис.7. Распределение Hsp90 у *Quasitetrastemma stimpsoni* и *Lineus alborostratus* после 4-часового теплового стресса. A–D — *Q. stimpsoni*; E, F — *L. alborostratus*. A, E, F — стенка тела; B — боковые нервные стволы; C, F — желудок; D — хобот.

Стрелки указывают на иммунореактивные эпидермальные гранулы, наконечники стрелок указывают на боковой нервный ствол и нейроны.

Обозначения: ct — кутис; d — дерма; ep — эпидермис; gep — гастроэпидермис; ln — боковые нервные стволы; m — мускулатура стенки тела; pge — эпителий хобота; st — желудок. Масштаб: A–D — 50 мкм.

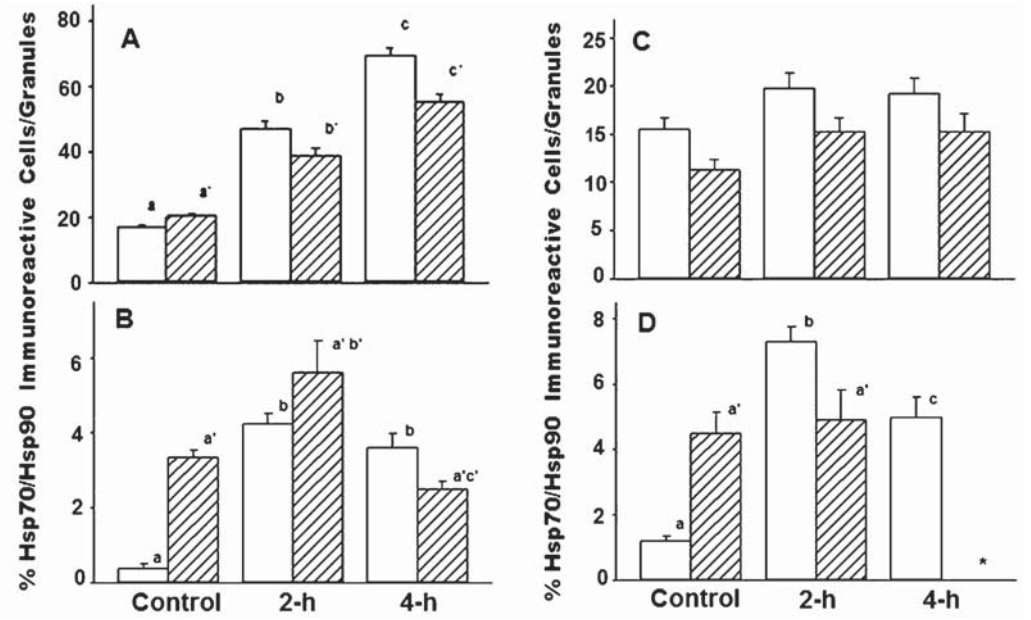

Fig. 8. Percentage (means ± SEM) of Hsp70 (open) and Hsp90 (hatch) immunoreactive cells in epidermal tissues of *Quasitetrastemma stimpsoni* and *Lineus alborostratus* and of the granules in 100 µm epidermis length (n=5) in *Q. stimpsoni* and in *L. alborostratus*.

A — percentage of Hsp70 and Hsp90 immunoreactive cells in epidermal tissues of *Q. stimpsoni*; B — percentage of Hsp70 and Hsp90 immunoreactive of the granules in 100 µm epidermis length in *Q. stimpsoni*; C — percentage of Hsp70 and Hsp90 immunoreactive cells in epidermal tissues of *L. alborostratus;* D — percentage of Hsp70 and Hsp90 immunoreactive of the granules in 100 µm epidermis length in *L. alborostratus*.

Abbreviations: The asterisk indicates completely-fused granules as a continuous layer. Different a–c and a'–c' represent significant differences among the Hsp70 and Hsp90 immunoreactivities, respectively.

Рис. 8. Процент (среднее ± ошибка среднего) Hsp70 и Hsp90-иммунореактивных клеток в эпидермальных тканях *Quasitetrastemma stimpsoni* и *Lineus alborostratus* и гранул на 100 мкм эпидермиса (n = 5) у *Q. stimpsoni* и *L. alborostratus*.

A — процент Hsp70 и Hsp90-иммунореактивных клеток в эпидермальных тканях *Q. stimpsoni*; B — процент Hsp70 и Hsp90-иммунореактивных гранул на 100 мкм эпидермиса у *Q. stimpsoni*; C — процент Hsp70 и Hsp90-иммунореактивных клеток в эпидермальных тканях *L. alborostratus*; D — процент Hsp70 и Hsp90-иммунореактивных гранул на 100 мкм эпидермиса у *L. alborostratus*.

Обозначения: Звездочка показывает полностью слившиеся гранулы в виде непрерывного слоя. Различные a–c и a'–c' представляют существенные различия между иммунореактивностью Hsp70 и Hsp90, соответственно.

thermal stress in *L. alborostratus*. This response by the nemertean demonstrates the possible role of this protein as a defense mechanism against thermal stress. Hsp70 is the most prominent protein induced by elevated temperatures and plays a central role allowing cell survival during and after thermal stress (Parsell, Lindquist, 1993).

In thermally stressed *Q. stimpsoni*, Hsp70 and Hsp90 were barely detectable in this study. However, Hsp70 and Hsp90 immunoreactivities were detected in epidermal cells and in the supra-epidermal granules. These results reveal the variability of physiological response to thermal stress as this nemertean species is abundant intertidally and often lives together with *L. alborostratus*. Both species, however, differ in body sizes (*Q. stimpsoni* ~3–5 cm long; *L. alborostratus* ~30–40 cm long). Additionally, the internal morphology of the hoplonemertean *Q. stimpsoni* lacks the glands in subepidermal layer which consists only of extra-cellular ma-

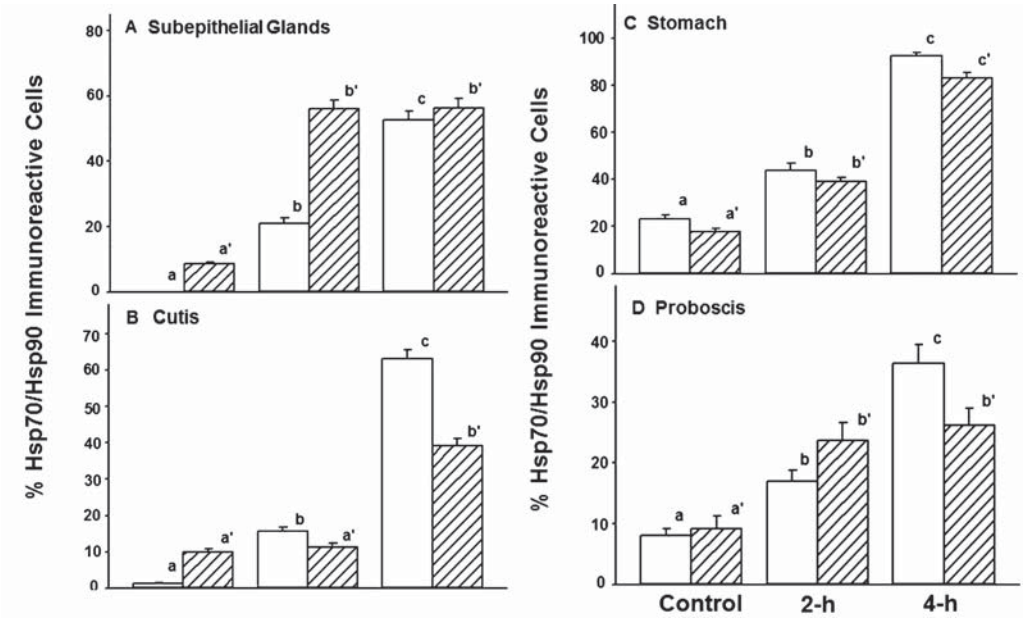

Fig. 9. Percentage (means ± SEM) of Hsp70 (open) and Hsp90 (hatch) immunoreactive cells in the subepithelial glands, cutis, stomach and proboscis of *Lineus alborostratus*.
A — the subepithelial glands; B — cutis; C — stomach; D — proboscis.
The asterisk indicates no immunoreactivity detected in all of the samples granules as a continuous layer. Data for these four tissues for *Q. stimpsoni* are not shown since only few cells from the stomach and proboscis displayed Hsp90 immunoreactivity at 4-h treatment. Different a–c and a'–c' represent significant differences among the Hsp70 and Hsp90 immunoreactivities, respectively. Since some of the *L. alborostratus* had lost their proboscises, only five specimens were analyzed for each Hsp.
Рис. 9. Процент (среднее ± ошибка среднего) Hsp70 и Hsp90-иммунореактивных клеток в субэпидермаальных железах, кутисе, желудке и хоботе у *L. alborostratus*.
A — субэпидермаальные железы; B — кутис; C — желудок; D — хобот.
Звездочка указывает на отсутствие иммунореактивности во всех образцах, где гранулы образуют непрерывный слой. Данные для этих четырех тканей у *Q. stimpsoni* не показаны, так как только несколько клеток в желудке и хоботе Hsp90-иммунореактивны после 4-часового теплового стресса. Различные a–c и a'–c' представляют существенные различия между Hsp70 и Hsp90-иммунореактивностью, соответственно. Так как некоторые из *L. alborostratus* потеряли свои хоботы, только пять образцов были проанализированы для каждого Hsp.

trix. However, in the heteronemertean *L. alborostratus*, this layer is well-developed with numerous and abundant glands which in this study showed their immunoreactivities to both Hsps. In Peter the Great Bay the reproductive season of *L. alborostratus* occurs in June with water temperatures 10–15°C, while *Q. stimpsoni* spawns in July–September with water temperatures 20–22°C (Chernyshev, 2014). The differences in Hsp response to thermal stress could be attributed to *Q. stimpsoni* being a more thermophilous species than *L. alborostratus*.

Our study showed immunoreactivities of Hsp70 and Hsp90 in both nemertean species were predominantly localized specifically in the body wall and stomach epithelia. Since nemerteans have no specialized protective structures (cuticle, exoskeleton), their body wall is the only structure that provides a barrier with respect to movement of water and solutes, as well as to environmental factors, e.g. increased temperature. In this study, differences in the distribution of Hsps were observed in the body wall. In *L. alborostratus*, Hsp70 and Hsp90 were detected in the epidermal cells and cutis, while in *Q. stimpsoni* these proteins were only found in the epidermal cells. Additionally, high immunoreactivities were detected in the supra-

epidermal granules, especially after 2 h in both ribbon-worm species.

In this study, the two ribbon-worm species showed accumulation of Hsp immunoreactive granules on the epidermal surface. High numbers of these granules were detected for *Q. stimpsoni* at the 4-h interval while complete-fusion of the granules was observed for *L. alborostratus*. Interestingly, the subepidermal glands were quite active in *L. alborostratus*. This observation appears to demonstrate that the epidermal and subepidermal glands may be a rapid initial response to thermal stress as indicated by production of supra-epidermal granules which have fused quickly. Moreover, when irritated, *Q. stimpsoni* and especially *L. alborostratus*, release a large amount of mucus (A.V. Chernyshev, pers. observ.), which may play a protective role. Okazaki *et al*. (2001) also observed copious mucus secretion when *Paranemertes peregrina* were thermally stressed.

Hsps are reported to be important for the stability of the membrane lipids (Tkáèová, Angelovièová, 2012). Pratt (1997) reported that Hsp90 functions in formation of steroid receptor complexes in membranes. Presence of Hsp on cell surfaces have been observed in mammalian neurons (Sidera *et al*., 2004; Sidera, Patsavoudi, 2008) and in plasma membranes of tumor cells (Hantschel *et al*., 2000; Eustace *et al*., 2004; Capello *et al*., 2006). Interactions of Hsps with external domains of receptors and enzymes on the surfaces of tumor cells and their participation in signal transduction in various types of cells have previously been reported (Eustace *et al*., 2004; Sidera, Patsavoudi, 2008).

Accumulation of extracellular Hsp70 and Hsp90 on nemertean epidermal surfaces could be a part of initial intracellular responses activated by environmental stressors, such as hyperthermia. Perhaps the low Hsp concentrations detected in the immunoblots for *Q. stimpsoni* could be attributed to these proteins being extruded outside of the ribbon worms during its response to thermal stress. These Hsps could have been contained in the mucus or even lost to the surrounding water.

Thus, the role of Hsps in membrane viability and stability could be triggering mucus secretion which might be an important mechanism in invertebrates without protective coverings.

Therefore, these Hsps in soft-bodied nemerteans may have evolved in this spiralian taxon as a protective mechanism commonly shared from phylogenetically distant groups, e.g. bacteria, plants to mammals.

Another effective barrier in nemerteans might be the wall of their digestive system. This study showed increased Hsp70 and Hsp90 immunoreactivities in the stomach epithelium in thermally-stressed nemertean species. Similar results have been reported for vertebrates (Lalles, David, 2011). In the control *L. alborostratus*, Hsp90 immunoreactivity was observed in a small number of epithelial cells of the stomach but substantially increased under 2- and 4-h thermal stress. In *Q. stimpsoni,* Hsp90 immunoreactivity were detected in the epithelial cells and the lumen of the stomach and intestine only after 4-h thermal stress. Moreover, in *L. alborostratus* exposed to maximum 4-h thermal stress, Hsp70 accumulated in the stomach. Increased concentrations of Hsp70 have been reported to protect epithelial cells of mammalian digestive tracts against toxins and ulcerogenic agents under thermal stress (Watanabe *et al*., 2004; Oyake *et al*., 2006; Pierzchalski *et al*., 2006; Poltronieri *et al*., 2008; Zhong *et al*., 2010). A few studies have shown that Hsp can be released from various types of cells of both vertebrates (Hightower, Guidon, 1989) and invertebrates (Tytell *et al*., 1986) into the extracellular matrix. The protective mechanism involving extracellular Hsp in invertebrates has not been previously studied. In mammals, extracellular Hsp has been reported to interact with the neighboring cells and protect them from death (Houenou *et al*., 1996).

All nemerteans characteristically possess an eversible proboscis lined by a glandular epithelium. For the control *L. alborostratus,* Hsp70 and Hsp90 immunoreactivities were observed in a small number of epithelial cells of the proboscis but increased substantially during subsequent exposure to thermal stress. In the

proboscis of *Q. stimpsoni,* Hsp70 immunoreactivity was absent, but isolated cells with Hsp90 immunoreactivity was only detected after 4-h thermal stress. These differences could be the result of variable feeding behaviors exhibited by *L. alborostratus* and *Q. stimpsoni*. The nemertean proboscis is principally used in prey capture. In all heteronemerteans (e.g. *L. alborostratus*), the everted proboscis coils around the prey with its epithelium in close contact with prey tissue. In the hoplonemerteans (e.g. *Q. stimpsoni*), the proboscis possesses a terminal stylet to pierce the prey with its epithelium infrequently used in prey capture. Thus Hsps in the proboscis of *L. alborostratus* may be more numerous and sensitive to activation in responding to stresses in dealing with the resistance of its prey. Moreover, some of nemerteans exposed to longer duration of thermal stress lost their proboscis during the experiment.

In this study Hsp70 and Hsp90 were detected in the tissues of two different nemertean species under thermal stress. However pathological changes in nemertean tissues were not observed. These two Hsps appeared to be involved in maintaining and protecting the integrity of cellular and physiological functions. Although we only investigated Hsp70 and Hsp90, future studies could determine if other Hsps, e.g. Hsp40, Hsp60, and Hsp110 are also involved in modulating the Hsp70 and Hsp90 thermal stress responses. The role of Hsps has evolved in this group of soft bodied ribbon worms in their adaptation to a whole range of adverse environmental factors, including thermal stress.

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