Age-related changes in the anti-radical defense system in the tissues of Yesso scallop *Mizuhopecten Yessoensis* (JAY1857)

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**Abstract.** The glutathione level and the total oxyradical scavenging capacity in the digestive gland and the gills of the Yesso scallop *Mizuhopecten yessoensis* individuals of different ages (2-7 years) have been studied. The research revealed the tissue-specific high glutathione content in the gills of the scallop and the age-related decline tendency of glutathione level in the digestive gland. TOSC as a complex antioxidant capacity indicator is less tissue-specific but also depends on age.

1. Introduction

Among other scallops of the Pectinidae family, the Yesso scallop *Mizuhopecten yessoensis* (Jay, 1857) is a unique model for studying the antioxidant system capacity changes caused by the changing environmental factors. *M. yessoensis* is a stenobiont organism sensitive to salinity, lack of oxygen and environment temperature fluctuations (Yesso Scallop, 1986). *M. yessoensis* prefers well-aerated regions with a stable strong current, where the water temperature does not drop below -1.5 °C in winter and does not rise above 18-20 °C in summer, and the range of dissolved oxygen concentration required for normal functioning of the scallop is limited to 5 - 9 ml/L⁻¹ (Primorskii grebeshok, 1986) [1]. Oxygen consumption may vary depending on the physiological condition of the scallop, the season and the habitat conditions. For this reason, in the scallop tissues and, first of all, in the scallop gills, the speed of the O₂-related metabolic processes is much higher than those of other bivalves. Therefore, the functioning specificity of the antioxidant system of *M. yessoensis* is directly adjusted to the oxygen content in the environment.

In the aerobic organisms' energetics, oxygen plays a key role, being one of the essential limiting factors of their activity. Upon entering the cell, the reactive oxygen molecule gets involved in the oxidation-reduction free radical-producing reactions and, in the regular situation, in the cascade of reactions, they are disposed or converted into other non-toxic substances. The oxyradical generation level may significantly grow under the influence of chemical compounds of different nature or physiological and biochemical changes the marine invertebrates go through during their life. The result of these processes is the uncontrolled generation of the reactive oxygen species (ROS) aimed at proteins, lipids, and nucleic acids. The system in charge of the ROS disposal and conversion into less reactive forms in the cells is the antioxidant (AO) system. The AO system of the organism is involved in defending the cells from the oxyradical toxic effect. The cell antioxidant system, conventionally divided into the anti-peroxide...
and anti-radical systems, is represented by specifically adjusted enzymes, vitamins, and other low-molecular compounds. Studying the individual antioxidant components, it is not always possible to make up a general picture of the changes occurring in the AO system as a whole, and, all the more, to use these data to assess the general state of the organism. For example, the studies of glutathione (GSH) levels of the marine invertebrates are mostly focused on their functional roles in the anti-radical reactions and xenobiotic detoxication of the scallops [2-7]. This is explained by the fact that a living organism is a sophisticated organized system, capable of maintaining and regulating its optimal level of various antioxidants (enzymes and low-molecular antioxidants). In such a system, a change in one element of the complex AO defence system may be compensated by synthesizing another defensive component. Moreover, antioxidants are considered to be able to function cooperatively, providing a higher cell defensive potential than any of the components could provide on its own [8]. This cooperative defence of the cell from the free radical impact determines the overall anti-radical status of the cell referred to as TOCS, Total Oxyradical Scavenger Capacity [9], or IAA, Integral Anti-radical Activity [10].

The anti-radical mechanisms present in the cell are believed to begin their work at the earliest stages of the organism development, establishing, therefore, a strict balance between the intensiveness of the oxidation processes and the AO defence system activity. Therefore, the antioxidant component level of the cell indicates the adaptation capacity of the organism both to the changing environmental conditions and the physiological changes occurring inside the organism. Taking the aforementioned physiological and ecological specificity of M. yessoensis into account, it becomes especially interesting to study the age criterion effect on the antioxidant system functioning in this bivalve molluscs. There are not so many published works dedicated to the questions of interconnection between the age and the biological response to the oxidizing stress of the Pectinidae [11, 12]. For this reason, the objective of this work is to study the age-related changes in the anti-radical defence system activity and the reduced glutathione level (GSH) in the digestive gland and gill tissue of the Yesso scallop Mizuhopecten yessoensis.

2. Materials and methods
The M. yessoensis individuals were taken from the Shirokaya Bay (42°52′36.1″N 132°26′35.2″E) of the Peter the Great Bay in the Sea of Japan (figure 1) in July. For the research, the following age group of the scallops were selected: 1, 2, 3, 4, 5, 6 and 7-year-old individuals. Every age group consisted of ten individuals. The captured scallops were transported to the laboratory for acclimation for 7 days. The M. yessoensis individuals were kept in the aquariums with aerated circulating water under a constant temperature. After the acclimation period, the digestive gland and the gills of every Yesso scallop M. yessoensis were taken out on ice for further biochemical studies. The tissues were homogenized in 0.02 mol Tris-HCl buffer of pH 8.5 with 1 mmol PMSF (protease inhibition) and 10 mmol DTT (to prevent the sulphydryl groups of the cysteine residues of the protein amino acids from oxidation.

2.1. TOSC
To evaluate the general antioxidant potential level of the tissues, the Total Oxyradical Scavenger Capacity (TOSC) was used [9, 10]. The method is based on determining the sample capacity to neutralize the hydroxyl radical generated in vitro in the Fe-ascorbate system. As the hydroxyl radical acceptor, the 2-hydroxybenzoic acid (salicylic acid) was used. The salicylic acid oxidation products (2,4-dihydroxybenzoic acid) were registered with the reversed-phase high-performance liquid chromatography (RP-HPLC, Knauf). The hydroxyl radicals were generated with the Haber-Weiss reaction in vitro (1.0 ml) containing 2 µmol of FeCl₃, 4 µmol of ethylenediaminetetraacetic acid (EDTA) and 200 µmol of acetylsalicylic acid in 0.1 mol of the phosphate buffer pH 7.4 in the presence of 0.7 mmol of salicylic acid. The reaction was initiated by adding Fe³⁺ ions. The derived compound was incubated for 60 min under 35°C. The acquired hydroxyl radicals oxidized the salicylic acid to produce 2,4-dihydrobenzoic acid – DHBA (reference samples). When adding the homogeneous aliquot of the studied tissues to the incubated mixe, the DHBA level was decreased proportionally to the introduced homogenate amount. The results were recorded as a percentage of the hydroxyl radical reduction in the
biological sample compared to the reference sample. The higher the index value (in per cent), the higher is the organism’s AO resistance to the oxyradical exposure [10].

Figure 1. Capturing site of the Yesso scallop *M. yessoensis* individuals of different age groups (Shirokaya Bay, Peter the Great Bay, Sea of Japan 42°52'36.1"N 132°26'35.2"E).

2.2. Glutathione detection
The glutathione detection method is based on the registration of the coloured product produced in the reaction of the cysteine thiol group with Ellman's reagent (dinitrobenzoic acid) [13].

To the studied tissue homogenate, 30% solution of trichloracetic acid (TCA) was introduced to the final concentration of 5%. The product was thoroughly mixed and placed into the temperature of -20 °C for one hour. Then the precipitation was centrifuged for 10 minutes under 5000 g. To the supernatant aliquot (0.1 ml), 2.0 ml of 0.6 mmol solution of dithionitrobenzoic acid (DTNB) prepared with 0.2 mol phosphate buffer, pH 8.0 was introduced, diluting the volume to 3 ml with the same buffer. The complex generation was registered with the Shimadzu AA-1650PC spectrophotometer at \( \lambda = 412 \) nm. The quantitative calculations were based on the reference glutathione solutions prepared with the molar extinction coefficient \( \varepsilon_{412} = 13600 \).

The statistically relevant differences between the experimental and reference values were found with the ANOVA simultaneous component analysis. All conclusions were made at the minimum relevance level of 5%.

3. Results
The comparative study revealed that TOSC and the reduced glutathione level are tissue-specific indexes that depend on the age of the individual.
In the digestive gland, the glutathione level increases with the age in the range from 2 to 3 years old (from 2.5 to 7.5 µg/ml). After the age of three years old, in every studied age group this value decreases (figure 2).

![Figure 2. Age-related changes in the glutathione content in the digestive gland and gill tissues of M. yessoensis.](image)

By the age of seven years, the glutathione level drops lower than that of the two-year-old scallops (from 2.5 to 2 µg/ml). Similar results were acquired in the gills of the studied scallops: the glutathione level increased from 2.5 µg/ml for the two-year-old scallops to 16 µg/ml for the three-year-old individuals. However, the glutathione level is reduced from three to five years old (to 3.85 µg/ml) and then increased by almost two times and then remains at the approximately same level for the 6 and 7-year-old scallops (7.6-7.9 µg/ml). For the 7+-year-old individuals, these values are three times higher than those of the two-year-old scallops.

The TOSC results demonstrated that its value for the digestive gland and gill tissues also depends on the age and tissue type (figure 3).

Compared to the gills, the digestive gland cells of the 2+, 5+ and 7+-year-old individuals show higher radical scavenger capacity values (figure 3). Interestingly, the AO system's hydroxyl radical inhibition capacity (52%), typical for the age of two years old, is later replaced with the reducing TOSC values of three and four-year-old scallops. The greatest reliable TOSC value in the digestive gland was found in the five-year-old scallops (71% inhibition). In the age group from 5 to 7 years old, the total radical scavenger capacity is reduced: for six-year-old individuals, to 60%, and for seven-year-old individuals, to 42%. In the age group of 3+, in the gills cell, the tendency to increased AO system capacity was found (TOSC level increased from 45% to 73%), and then this capacity reduces: in the 4, 5, 6, and 7-year-old individuals the TOSC was noticed to reduce (63.6%; 33.55%; 32%; 31%), which is a tendency similar to that of glutathione in the digestive gland.
4. Discuss

For the bivalve molluscs, the energetic and plastic demands for the growth and functioning of the cells depend on such environment conditions [14-16] as salinity, temperature, oxygen etc. In this regard, being a stenohaline and stenoxyle organism, the Yesso scallop *M. yessoensis* is especially interesting for studying the age-related specificities of the biochemical reactions in charge of acclimation to the environment. The strategy of biochemical adaptation developed by *M. yessoensis* in the course of the evolution is closely related to the specific concentration of oxygen in the environment and, therefore, to the concentration of oxygen consumed by the organism. As this bivalve representative is capable of free and active motion, it has a higher metabolism and, therefore, finds itself in the active need for oxygen and is capable of bearing only short-term oxygen shortages (due to the accumulated metabolism products needing oxidation). This is why the need for a sufficient and stable concentration of oxygen in the environment may be the reason for high ROS generation value in the Yesso scallop tissues.

4.1. GSH

Despite the age-related decline of the GSH value (from 3+ to 7 years old), we noticed that the tripeptide concentration in the scallop gills is higher than in the digestive gland cells. In our opinion, such tissue-specificity of the Yesso scallop may be caused by the structural and functional peculiarities of its tissue structure. The gills are an organ located in the ventilated mantle cavity of the scallop, contacting with the oxygen-saturated water [17]. The gill epithelium is not thick, which is an essential condition for sufficient gas exchange between the hemolymph and the mantle fluid. This is why the gills bear a large oxidizing load, as they are exposed to a large external oxygen load (environment oxygen), being the first to contact with various toxic compounds of the environment [10, 17]. Taking the high activity of the AO enzymes, especially catalase [18] in the Yesso scallop gills and the high GSH level (figure 2), we may conclude that the activity of the AO system in the gill cells is one of the evolved adaptation mechanisms used by the organism to regulate its functions to avoid excessive shifts under the influence of external factors. We suppose that the found abrupt rise in the glutathione level in the gills and the
digestive gland of 3-4-year-old *M. yessoensis* may be caused not only by the physiological age-related changes, intensiveness of metabolism involving active oxygen consumption by the scallops but also by the accumulation of such microelements as cadmium and iron. It should also be noticed that even in the relatively clean water areas, *M. yessoensis* may accumulate a high concentration of metals in their soft tissues and, first of all, in the digestive gland [19-21]. For example, the cadmium and iron content in the digestive gland of *M. yessoensis* is 36 and 10 times higher, respectively, than that of the mussel *Crenomytilus grayanus* [20, 21]. With the age, metal concentration in all the soft tissues and certain organs of the molluscs is gradually accumulating, therefore increasing the load on the AO defence, and the glutathione system in particular. It is a common fact that in the oxidation-reduction metabolism of the cell, a material role is played by glutathione, which manifests its properties as an antioxidant and acts as the direct oxyradical scavenger or acts as a co-factor for the AO enzymes. Moreover, high GSH concentration may be caused by its active ability to bond accumulated metals [18, 22]. Therefore, the decline in the GSH level in the digestive gland cells of the 5-7-year-old individuals may indicate the physiological aspect of the phenomenon, i.e. the total AO status decline is obviously related to the decline of the overall antioxidant enzyme activity, and, therefore, the decline in the reduced glutathione level which cannot be recovered from the oxidized form with the glutathione reductase. Besides, glutathione forms pools with the metals, the content of which grows with the scallop age; therefore, the reduced tripeptide concentration in the cell decreases. This way, as the activity of such cells is growing in such stressful situations as exposure to xenobiotics, puberty period etc., the glutathione level in the cell is rising. However, as the speed of the age factor-caused metabolic processes reduced, the total antioxidant scavenger capacity also decreases and, consequently, so does the glutathione level in the digestive gland cells.

4.2. TOSC
Depressing activity or content of the given AO system components is one of the commonly known properties of the oxidation stress, and the integral TOSC indicator demonstrates the change in the activity of some AO enzymes and the antioxidant level in the stressed condition, as well as the sustainability, sensitivity or predisposition of the organism to the oxidation stress.

One of the cell oxidation stress development patterns is to produce free radicals in the presence of transition metals (iron, copper) as well as cadmium. As mentioned above, the Yesso scallop accumulates a high concentration of cadmium and iron in its organs and tissues. Iron is a high-reactive and potentially toxic element found in the living cell; accumulation and storage of iron in the marine animals' cells are controlled by the transport and binding proteins [23]. Besides these proteins, the cells have the cellular labile iron pool (Fe^{2+} and Fe^{3+}) combined with different chelating agents of low molecular weight, making up the minor part of the overall iron content [24]. Supposedly, the excesses of Fe^{2+} and Fe^{3+} in the cell stimulate the development of the oxidation stress by means of iron-caused conversion of the superoxide radical and hydrogen peroxide into a hydroxyl radical (Haber-Weiss reaction) [18, 25]. Unlike iron, the cadmium accumulates in the cells cannot directly participate in the ROS production through the oxidation-reduction reactions [23, 26]. According to the experimental data at hand, the Cd^{2+} ions depress the enzymes and non-enzymes lipid peroxidation initiation processes, which means that the given metal may stimulate the oxidation stress by depressing the enzyme antioxidant defence [27, 28].}

As a result of the research, it was concluded that the scallops' ability to inactivate the hydroxyl radicals in the digestion gland cells does not drop dramatically with the age, but reaches its maximum by the age of 5+ and generally remains at a high level. We explain it with the active accumulation of the microelements in the digestion gland, mainly iron and cadmium, together with the evolving acclimation of the antioxidant defence system to this specificity of the scallop. The AO defence mechanisms of the gills and the digestion gland are different in their strategic trajectories. The gill cells are continuously exposed to the aggressive oxygen attacks, while the digestive gland cells are more protected from the oxygen molecules; therefore, in this organ, the microelements are accumulated in a gradual manner and at a slow pace.
Besides the metals influencing the oxidation stress development, the accelerated free radical generation in the cells may also be initiated by the physiological changes in the organism. In natural *M. yessoensis* populations, the individuals reach puberty by the age of three years old (Primorskii grebeshok, 1986), which is the period of active growth and maturing of the gametal cells in the gonads. This period is specific with the higher rates of metabolism, oxygen consumption and free radical generation in the cells. The power law dependence of the oxygen consumption speed on the mass of the metabolically active body parts of the scallop has been formulated, where the larger is the soft tissue weight, the higher is the exchange speed. Thus, for the age group of 3-5-year-old scallops, under the water temperature of 20°C the exchange speed reaches 6.02 mg/h per individual, while for the 1-2-year-old scallops this indicator equals to 0.86 mg/h per individual (Primorskii grebeshok, 1986). This way, the scallop reaches its physiological and biochemical stability by the age of 3+ - 4+, after it has been through its second spawning and the organism has ultimately readjusted itself from the juvenile stage to the continuous reproductive cells generation. Then it appears rational to suppose that the amount of the peroxide oxidation products and other indexes pointing out at the oxidation processes in the organism may be different from one age group to another. As we found, the 2-4-year-old individuals manifest the highest level of anti-radical activity and powerful antioxidant defence.

The oxidation stress is related not only to the intensive growth and reproduction period but also to the ageing process. The studies of [12] based on *A. ventricosus* scallop demonstrated that the oxygen consumption speed and the oxidation stress biomarkers depend greatly on the somatic growth. In the intensive growth and reproduction periods, the oxidative damage accumulation and the antioxidant defence reduction were also demonstrated by the organisms of various organization levels, such as mammals, birds, and a number of bivalve molluscs [29-33]. It is a known fact that the higher is the age of a biological object, the lower is the metabolic rate, the lower is the oxygen consumption and, as a result, the lower is the ROS production. However, this assumption may not be right, as one of the ageing reasons is the free radical mechanism hypothesis that claims that the older is the organism, the higher is the free radical generation rate [34-36]. It should be noted that the high level of the radical generation in the cell is not the generation speed, but the metabolism reduction rate and, as a result, the inability to use the ROS to the full extent for the successful functioning of the organism as a whole. In his research, Viarengo [3] notes the age-related reduction of the low-molecular antioxidants (glutathione and Vitamin E) in the digestive gland of the *Mytilus edulis L* mussel, associating this fact with the reduction of the antioxidant system component content in the mussel digestive gland and claiming that the combination of these facts causes severe oxidation stress. This is also aligned with the findings of our research. Therefore, the rate of ROS transformation to non-toxic forms is slowing down, consequentially, causing free radical accumulation.

**5. Conclusion**

From this research, it can be concluded, that in the evolution process *M. yessoensis* has developed its own strategy, determined by the adaptation of the anti-radical defence system to the habitat conditions. This strategy features a number of properties: increasing the low-molecular antioxidant (glutathione) synthesis rate in the highly exposed organs, particularly gills, and maintaining the common radical scavenging capacity in the tissues. This strategy enables the scallops to function and survive in the active oxygen consumption, adjusting to various age groups to ensure the survival of the species within the evolutionary-determined age limit.

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