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MOLECULAR MECHANISMS OF ANTIOXIDANT PROTECTIVE PROCESSES IN HONEYBEE *APIS MELLIFERA*

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20.1 INTRODUCTION

Features of the honeybee evolutionary development have defined a special role of oxidative stress and the molecular mechanisms of its regulation in the processes associated with homeostasis preservation by an individual and a bee family.

Normally, reactive oxygen species (ROS) are produced continuously and moderately in cells. A cell neutralizes them with the help of the antioxidant system and replaces damaged molecules. A level of ROS exceeding the protective capabilities of a cell can cause a serious damage, even the cell's death. Thus oxidative stress as the cost of an aerobic life is a constant cause or an important component of many problems for the organism.

Nevertheless, oxidative stress is actively used by insect organism as a defense mechanism to neutralize xenobiotics and fight against pathogens in the processes of morphogenesis and life span regulation. Some ROS can act as messengers through redox signaling. In this situation, the system of antioxidant protection of a honeybee provides a wide range of vital important functions, including both protection against oxidative damage and adjusting an individual's life span in the colony. Moreover, eusociality obtained by individual phylogenetic groups of insects determines many peculiarities of the functional use of ROS and the molecular mechanisms of their regulation in the honeybee organism, as well as significant restructuring in the functional orientation of genome expression.

This chapter considers the role of oxidative stress in the process of determining the preservation of homeostasis by both a honeybee organism and a colony.

20.2 ANTIOXIDANT SYSTEM OF HONEYBEE

20.2.1 Honeybee as Insect

In the process of aerobic organism evolution, complex antioxidant systems, including enzymatic and nonenzymatic components, have been formed to prevent oxidative damage. There exist primary and secondary antioxidant enzymes that affect ROS molecules directly or indirectly.

Defense against the destructive action of ROS is first provided by three groups of primary antioxidant enzymes by direct action. Superoxide dismutase (SOD) converts superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2), which is then neutralized by catalase. Peroxidases catalyze similar reactions in which H_2O_2 is reduced to H_2O with the use of reduced thioredoxin (TRX) or glutathione (GSH) as an electron donor.

Key components of the antioxidant defense system are stored in organisms of all evolutionary branches, but there are unique adaptations peculiar to individual groups [1]. In insects, as compared to vertebrates and

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other phylogenetic groups, genes encoding glutathione reductase (GR) and glutathione peroxidase (GPX) are absent. Their functions are performed by homologous genes encoding thioredoxin reductase (TrxR) [2] and thioredoxin peroxidase (TPX) [3]. In addition, insects have genes that encode enzymes of antioxidant defense that act as peroxidases: phospholipid-hydroperoxide GPX homologs with TPX activity (GTPX) [4] and glutathione *S*-transferases (GST) [5, 6]. Thus the group of secondary antioxidant enzyme in insects acting on ROS indirectly includes TrxR, which converts both TRX and GSH, and also methionine sulfoxide reductases (MsrA and MsrB), which are involved in protein reparation by catalyzing the TRX-dependent conversion of methionine sulfoxide to methionine [7, 8].

Insects have an open circulatory system, so a free flow of ROS in the body can not only lead to foreign agent destruction but also can be very dangerous for the insect organism. Simultaneous functioning of the systems producing and neutralizing ROS allows on the one hand stopping the development of infection and on the other hand preventing the insect organism from total intoxication.

20.2.2 Honeybee as Species

Coding of the complete genomes of several species of insects allows us to make a preliminary comparison. The honeybee genome has been found to evolve more slowly than the genomes of the fruit fly and mosquito. In this case, the honeybee genome with respect to genes involved in circadian rhythm, RNA interference, and DNA methylation has more similarities to the genomes of vertebrates than to the genomes of the fruit fly and mosquito. At the same time, compared to *Drosophila melanogaster* and *Anopheles gambiae*, there are fewer genes presented in the genome of *Apis mellifera* associated with innate immunity, detoxification proteins, and gustatory receptors but more genes associated with the odorous substance receptors [9–11]. There are absolutely unique genes responsible for collecting and processing nectar and pollen. New microRNAs have been discovered that are expressed depending on the stage of development and specialization of bees; this means that these microRNAs participate in the honeybee social diversification [12]. To date, 39 genes coding 10 groups of antioxidant proteins have been identified in the honeybee genome (Table 20.1).

Special attention is paid to vitellogenin, the glycolipoprotein egg yolk precursor, involved in the development of reproductive functions of an insect female and a nutrient of the bee brood, which performs an antioxidant function in the honeybee organism [23–26]. Antioxidant properties of vitellogenin are stipulated by its Zn-binding capacity [22] and preferential oxidative carbonylation under oxidative stress in bees [23]. With respect to these properties, vitellogenin is compared to Cu/ZnSOD, key metal binding antioxidant enzymes also undergoing preferential carbonylation [27], and serum albumin, metal binding proteins that can function as free radical acceptors and reduce levels of oxidative markers such as protein carbonylation [28].

TABLE 20.1 Antioxidant proteins of the honeybee

Antioxidant proteins	Antioxidant Functions	References
Superoxide dismutase: mitochondrial MnSOD cytoplasmic Cu/ZnSOD	Reduction of $O_2^{\bullet -}$ formed in mitochondria to $O_2$and H_2O_2	1, 13, 14
Catalase: cytoplasmic extracytoplasmic (in honey)	Reduction of $O_2^{\bullet -}$ to $O_2$and H_2O_2	1, 13, 15
TPX (peroxiredoxin)	Reduction of H_2O_2 with the use extracytoplasmic (in honey) of TRX as a donor of $\bar{e}$	1, 3, 16, 17
GTPX	Reduction of H_2O_2 and organic hydroperoxides	1, 4
TrxR	Transfer of reducing equivalents from NADPH to TRX and GSH disulfide with formation of powerful intracellular antioxidants—thiol-based reductants	1, 2, 18
TRX	Preservation of redox homeostasis homologs of TRX of the cell	1, 2, 18
Glutaredoxin (GRX) homologs	Preservation of redox homeostasis of the cell	1, 2, 18
GST	Metabolism of xenobiotics and protection against peroxidative damage	1, 5, 6, 13, 15, 17, 19–21
MsrA and MsrB	TRX-dependent reduction of methionine sulfoxide to methionine, participation in protein repair	1, 7, 8
Vitellogenin	Predominant carbonylation	22, 23

20.3 ROS AS COSTS OF AN AEROBIC METABOLISM

The main sources of ROS in any organism are the mitochondria (respiratory chain), microsomal oxidation of xenobiotics, and phagocytosis.

20.3.1 Mitochondria

ROS as by-products of aerobic metabolism are being continuously formed in the cells of honeybees under normal physiological conditions, and they necessitate constant monitoring of the antioxidant system. Two main reactions take place with the formation of ROS in the mitochondrial respiratory chain: the formation of $O_2^{\bullet -}$ from O_2 and its dismutation (disproportionation) under the influence of MnSOD with the formation of H_2O_2. In addition to the reactions occurring in the electron transport network of the inner mitochondrial membrane, the source of ROS is the oxidative deamination of biogenic amines with the formation of H_2O_2 under the influence of monoamine oxidases localized in the outer membrane of mitochondria. This reaction contributes significantly to the creation of a stable concentration gradient of ROS between the mitochondrial matrix and cytosol [29, 30]: The concentration of $O_2^{\bullet -}$ in the mitochondria in the norm is 5–10 times higher than in the cytosol and nucleus. Exceeding this level, and hyperproduction of H_2O_2, being relatively a long-lived molecule that easily diffuses through the mitochondrial membranes, creates the preconditions for the oxidative damage to mitochondrial matrix. According to some researchers, the intensive formation of H_2O_2 in mitochondria leads to disruption of intermolecular interactions and damage of the mitochondria inner membrane, as has been shown in studies of mitochondria isolated from muscle tissue of synanthropic flies in model systems generating ROS [31, 32]. Mitochondrial DNA is the most vulnerable to the damaging effect of ROS because it is in close proximity to the ROS-generating sites and is not protected, like the nuclear DNA, by histone proteins. The extreme sensitivity of the mitochondrial DNA to the damaging effect of ROS may lead to an increase in the number of mutations and the consequent suppression of aerobic respiration, since mitochondrial DNA encodes carrier proteins of the electron transport network.

20.3.2 Microsomes

Another source of ROS in the honeybee organism is the microsomal oxidation of xenobiotics. Microsomes contain enzymes of the system of cytochrome *P*450 (CYP), which catalyze polyvalent oxidation of xenobiotics with simultaneous generation of $O_2^{\bullet -}$ and other ROS. Two phases are distinguished in the process of xenobiotic clearance: the introduction of polar groups with the help of the CYP hydroxylase system and the conjugation of molecules with water-soluble ligands. Both processes are used to eliminate foreign components from the internal environment of an organism. The CYP hydroxylase system includes flavoproteins and a family of hemoproteins, which are localized on the cytoplasmic side of membranes of the endoplasmic reticulum. Different isoforms of CYP are involved in metabolism of various xenobiotics [33, 34]. Two groups of CYP are distinguished: The first is involved in the metabolism of endogenous substances; the second is induced by exogenous agents. The conjugation processes are often catalyzed by UDP-glucosidase, sulfotransferase, and GST. Glucuronidation is the major form of conjugation for agent detoxification. Sulfation usually provides a lowering of toxicity and acceleration of xenobiotic clearance. The GST reaction is important for neutralization of unstable electrophilic molecules. Microsomal GST is closely linked with the CYP system, which contributes to rapid inactivation of active metabolites produced during the metabolism of xenobiotics.

20.3.3 GST of Honeybee

The genome of the honeybee, in contrast to other insects, contains significantly fewer genes of xenobiotic detoxification protein. This may be due to the evolution of the hormonal and chemosensory processes and is a genetic payment for the highly organized eusociality of the honeybee. The most notable features of the honeybee are manifested in the coding of three important families of xenobiotic detoxification enzymes: Honeybees have only about half the genes of GST, CYP, and carboxyl/cholinesterases as other insects [35–37]. In particular delta and epsilon GST and CYP4, which contribute significantly to the binding of insecticides in other species, are insufficient. This deficit can contribute not only to honeybees' increased sensitivity to insecticides but to insufficient resistance to oxidative stress caused by incomplete microsomal oxidation of xenobiotics [38]. Foragers suffer from it more, because other members of the colony are relatively isolated from external influence in the hive.

Other insect GSTs have been assumed to play a minor role in protection against insecticides, only attenuating the effects of oxidative stress. However, lately it has been shown that GST, belonging to the Delta and Epsilon classes, are directly involved in the disposal of insecticides. The complete absence of class Epsilon enzymes in the honeybee and the presence of only one enzyme of class Delta GSTD1 [19, 39] may partly explain the

extreme sensitivity of this species to certain insecticides. Perhaps GSTD1 can serve as a universal mechanism for the protection of bees against harmful xenobiotic action. This enzyme is also involved in protection against oxidative stress. It is available in the spermatheca of the queen bee, and it is assumed to protect the sperm from oxidants.

In contrast to the lack of genes Delta and Epsilon GST, the honeybee has more genes of class Sigma. These enzymes were initially assumed to play an important role in the structural organization of muscle tissue [20], but enzymes of class Sigma GST turned out to have a high affinity to the products of lipid peroxidation and are localized in metabolically active tissues, such as those in the thorax muscle of flies. They are hypothesized to play an important role in protecting these tissues against oxidative stress by-products [21]. Omega, Theta, and Zeta classes of GS, are presented in bees as well as in other organisms. Probably, they play a key role in metabolic processes, in contrast to a predominantly detoxifying role of the Delta and Epsilon GST. This hypothesis is supported by the participation of the first in the degradation of products of tyrosine and phenylalanine metabolism and the importance of Omega GST in removing of S-thiol adducts from proteins [36]. Despite the fact that microsomal GST and cytosolic enzymes may play a similar role in the overall reaction of detoxification and protection from oxidative stress, they do not have structural similarities and are different in genetic origin [40].

Increase of activity of microsomal mixed-function oxidases and GST is recorded as the initial response of foragers to the action of pesticides and allelochemicals contained in toxic nectar, and the increase of the detoxifying activity depends on the age of worker bees [41, 42].

Thus the features of the cytochrome oxidase spectrum in the honeybee are mostly connected with the limitation of their function in xenobiotics metabolism and a great accentuation on hormones metabolism. As noted above, this aspect has a particular evolutionary significance for the honeybee.

20.3.4 ROS as Component of Immunity

Protection of insects against pathogenic microorganisms is carried out by phagocytic cells of hemolymph. The unlocked type of circulatory system promotes development of rapid generalized response to any influence. Metabolic activity, the so-called respiratory burst of phagocytic cells, is a source of formation in the NADPH oxidase reaction of $O_2^{\bullet -}$, triggering a cascade mechanism of ROS generation (Fig. 20.1). The latter have expressed microbicidal activity and are part of the cytotoxic

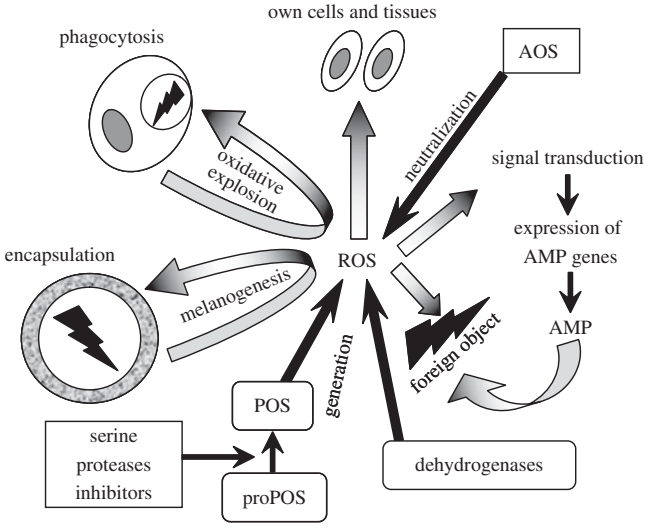

Fig. 20.1 Immune processes of the honeybee with ROS generation and neutralization. POS, phenoloxidase system; AOS, antioxidant system.

arsenal of the humoral immune system of honeybees [43, 44]. ROS are generated in the insect hemocytes with the participation of several enzyme systems—a phenoloxidase system (POS), a respiratory cascade, and a complex of dehydrogenases.

20.3.5 ROS and Phenoloxidase Cascade

Phenoloxidase (PO) is one of the oxidases responsible for the main part of the oxygen uptake during initiation of immune responses,as well as in morphogenetic processes of insects. The defeat of the honeybee by pathogens is accompanied by an increase of levels of proPO gene expression and PO activity [45–48]. In general, the power of the honeybee immune response and its ability to resist infection directly depend on the level of PO activity [49]. Quinone intermediates of melanin, $O_2^{\bullet -}$, H_2O_2, hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), and peroxynitrite ($ONOO^-$), involved in the cytotoxic reactions of insects in granule formation and encapsulation, are formed in large numbers during the realization of the PO cascade and melanogenesis in the hemolymph of insects [50–52].

From the immunological point of view it should be noted that biogenic amines (as mediators of stress reaction), as well as quinones, melanin, and other highly reactive metabolites formed during the activation of PO directly or indirectly participate in such defense reactions of insects as hemolymph coagulation and wound healing, encapsulation, granule formation, and destruction of the pathogen penetrated into the body by ROS. Components of the POS are also involved in the process

of immune recognition, carrying out cross-links of the foreign cell surface with the corresponding phagocyte receptors [53]. Uptake of foreign material by phagocytes is accompanied in both insects and vertebrates by an increase in oxygen consumption with active formation of ROS, which is analogous to the oxidative explosion [54, 55].

In addition, ROS generated during activation of PO and phagocytosis act as mediators of signal transduction, inducing gene expression of antimicrobial peptides (AMP) [51, 56, 57]. The presence of genes encoding proteins of the Toll-pathway of signal transduction in *A. mellifera* [58] suggests the existence of the same process in the honeybee.

However, if killer mechanisms of AMP are directed exclusively against bacterial and fungal cells, ROS have cytotoxic effects on all living cells, including cells of the insect organism itself. To reduce the cytotoxicity of ROS to the insect organism itself, its level is regulated by inhibitors of the proPO cascade, such as serine protease inhibitors [58] and components of the antioxidant system (AOS) [59, 60].

20.3.6 Interaction of Phenoloxidase and Antioxidant Systems

Correlation between functioning of the POS and antioxidant mechanisms in the defensive response of insects has been shown in a number of studies. The initial stage of infection in the honeybee has been established to be accompanied by activation of a PO cascade and inhibition of antioxidant enzymes activity [61]. The activity level of PO, catalase, and peroxidase in the honeybee imago changes under the action of the immunomodulator chitosan [62]. An analogous reaction of PO and AOS has been found under bacteriosis in lepidopterous larvae [52] and adults of Colorado potato beetle [63].

In the above-mentioned papers it is assumed that the temporary inhibition of the organism's own AOS may play an important role in antimicrobial immunity of insects through the production of ROS generated in the implementation of the PO cascade (see Fig. 20.2). However, a different explanation of these changes in enzymatic activity can be supposed. The PO cascade may act mainly on the pathways of the formation of quinones, serving as a trap for oxygen radicals and reducing the concentration of ROS in hemolymph and, therefore, the activity of enzymes neutralizing them [64].

Study of the interaction between different antioxidant enzymes under the action of honeybee pathogens has revealed a similar pattern. A marked differentiation between catalase and peroxidase functions has been shown in the hemolymph and gut [65, 66]. Utilization of H_2O_2 at the early stage of infection development is carried out by catalase, whereas in the later stages of pathogenesis peroxidase gets involved. An inverse correlation is observed in the development of these reactions.

20.3.7 Social Immunity

The above defense responses are components of individual immunity of the honeybee and are encoded by genes, being only one-third of the number of immune genes of nonsocial insects [58]. This payment for eusociality is compensated by effective social immunity, including health behavior and the secretion of antiseptic substances in the larval food and honey, as well as in the internal environment of the hive.

Glucose oxidase, catalyzing the oxidation of β-D-glucose to D-glucose with the formation of H_2O_2 and

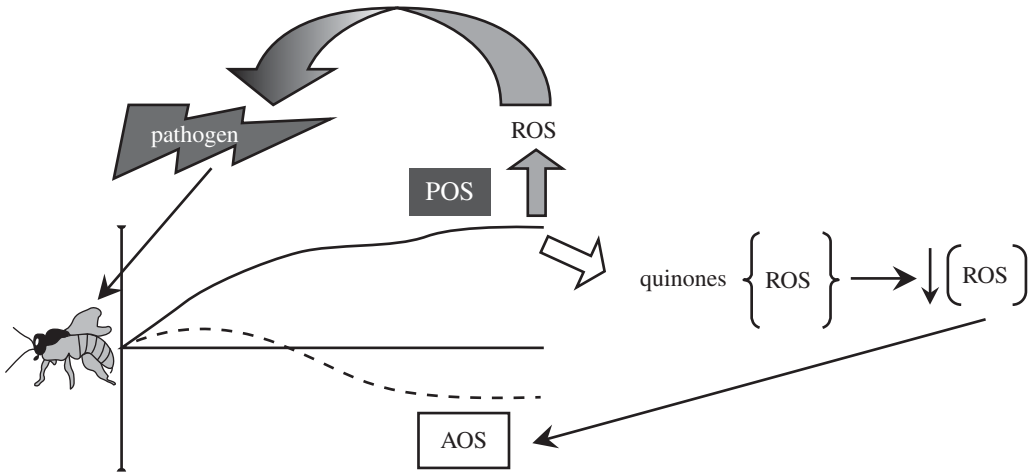

Fig. 20.2 Correlation of phenoloxidase and oxidative systems in honeybee defensive responses.

being a biochemical indicator of the level of social immunity of honeybees, plays an important role in providing of antiseptic properties of bee products [67]. Its activity is inhibited by the reaction product. The presence of this enzyme in honey with a large amount of substrate creates a relatively constant and self-regulating bactericidal system [68]. Importance of glucose oxidase in the group protection of honeybees is accentuated by the direct dependence of the colony strength on the level of this enzyme activity: H_2O_2 stipulates the antiseptic properties of the larval food and honey, inhibiting pathogen development [69, 70].

However, catalase activity, supporting H_2O_2 concentrations less toxic to the honeybee are found in honey. It is noteworthy that the genome of the honeybee contains only the gene of the intracellular catalase. The fact that the gene of catalase contained in honey is not integrated into the genome of the honeybee and extracellular catalase is found only in some bacteria and fungi suggests the expression of extracellular catalase of honey by endosymbiotic organisms [1].

Another component of the larval food—royal jelly—also contains highly active antioxidant substances that can reduce cadmium-induced genotoxicity and oxidative stress in mice significantly [71].

20.4 ONTOGENESIS AND LIFE SPAN

Oxidative stress and the molecular mechanisms of antioxidant protection of the honeybee motivate a particular research interest in connection with aging and longevity of animal organisms. The honeybee, because of the caste subdivision of the family, age-related functional specialization, and different life spans of the castes, is a natural model for gerontological research. According to the free radical theory of aging, the main reason for this process is the accumulation of ROS, oxidizing proteins [72], lipids, and DNA [73] and thus leading to DNA damage, degradation of membrane proteins, disruption of signaling pathways, and ultimately necrotic or apoptotic cell death [74].

Ontogenesis of the honeybee, which is a holometabolous insect, is characterized by a change of stages that are significantly different in morphology, physiology, and functioning. The transition from one stage to another is provided by major morphogenetic transformations occurring during metamorphosis and accompanied by the development of cellular and humoral reactions characteristic of anticontagious response [75]. Change in hormonal balance during periods of metamorphosis of the larvae and pupae, the appearance of the macrophagocytes in the hemolymph which are involved in the processes of histolysis, is also accompanied by the analog of the oxidative explosion and the induction of molecular mechanisms to restrain it. Changes in the activity of SOD, catalase,and GST in the development of different insects implies that the cellular antioxidants are involved in protecting cells against damage and regulation of redox levels in the process of insect morphogenesis and are hormone dependent [76–78]. In honeybees during the first 3 days of embryonic development the level of thioredoxin peroxidase and GST expression significantly increases, which indirectly confirms the assumption of an active generation of ROS due to the high oxygen demand in the developing embryo [17]. Catalase activity reaches its highest values in the intestine of fertilized queens, which is associated with their more active feeding and significantly greater life span [13].

Ontogenetic changes in the activity of immune factors of the honeybee are under hormonal control [22, 25, 79] and can be related to the varying ability of the honeybee for antiinfective protection at different stages of development. Evolutionarily established social relationships in a colony of honeybees exhibit age-related features of cellular protection in individuals. In the honeybee the total number of functionally active hemocytes increases at the larval and pupal stages of development [49], which confirms their active involvement in morphogenetic processes. During the maturation of adults immunocompetence of the hemocytes decreases regardless of bees' sexual and caste identity [80], and levels of POS and AOS activity increase [15, 49].

Relatively low resistance to pathogen action and short duration of life are beneficial in terms of viability of the bee colony. In accordance with the "law of 40 days," bees of the second summer generation (May–June) are grown in large quantities on a relatively poor diet for mass participation in the honey harvest. According to the Institute of Beekeeping in Russia, from June 20 to August 1 bees collect 90% of all feed stocks [81]. At the end of the main honey harvest the existence of worn-out "unemployed" foragers becomes unprofitable for the colony. Drone removal from the colony takes place at the same period.

In the period when worker bees start forage conservation, the intensive death of hemocytes is accompanied by increase of juvenile hormone titers and a simultaneous decrease in the vitellogenin level [23, 79] (see Fig. 20.3). It is remarkable that an active cell proliferation, accompanied by a fall in titer of endogenous juvenile hormone and increasing of vitellogenin levels, starts in the hemolymph of foragers that are forced to return to hive work [82].

In general, an increase in ROS generation and enhancement of antioxidant protection is associated with aging and transition of worker bees to foraging.

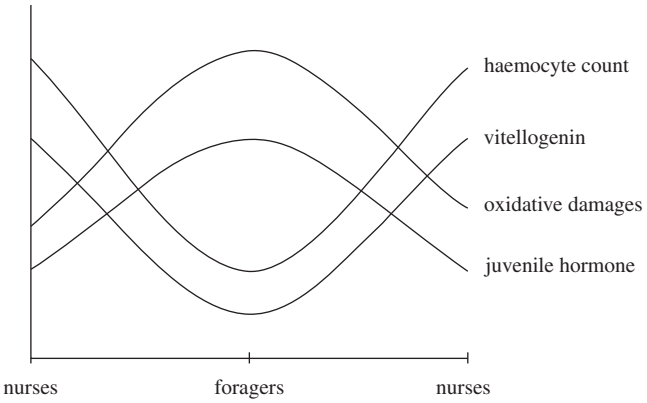

Fig. 20.3 Hormone-caused plasticity of worker bee defensive reactions.

Flight of foragers, as one of the most aerobically consumed types of activity, causes production of a large amount of ROS in the thorax muscle cells [83, 84], and a significant increase of Cu/ZnSOD content to neutralize $O_2^{\bullet -}$ [14]. In the midgut of worker bees the activity of catalase, GST, and microsomal oxidase of mixed function increases with aging [15, 41, 42], which reflects the conjugacy of detoxification and oxidative stress systems, being a result of foraging flight and associated contact with various pollutants.

The aging process is correlated with the accumulation of carbonylated proteins in the brain of foragers [23]. Carbonylation of proteins—the introduction of carbonyl groups in protein chains by direct oxidation or reaction with ROS—is one of the main oxidative modifications of proteins associated with biological aging [85].

Accumulation of carbonylated proteins is not the direct function of the chronological age of the worker bees, appearing in bees only after a long period of foraging activity [23]. Thus the processes of biological aging are associated not only with the real life expectancy of the honeybee, but with its behavior and functions performed in a colony.

Antioxidant enzymes also play an important role in the longevity of germinal cells. The sperm of drones is stored for a long period in spermatheca of a fertilized female, maintaining respiratory activity and thus running the risk of oxidative damage. However, sperm remains viable for many months, which is explained by a high activity of antioxidant enzymes in the reproductive tissues of the honeybee. Thus, in female spermatheca after fertilization, both the level of gene transcription of catalase and GST [19] and the activity of these enzymes greatly increase [13]. In addition, a high catalase activity in the spermatozoids and drone semen [13] and high levels of antioxidant-encoding transcripts in the reproductive organs of males [19] are associated.

20.4.1 Vitellogenin

The life span of the honeybee is closely associated with the functioning of vitellogenin, which has a positive effect on cellular immunity, preserving the integrity of proteins and increasing resistance of insects to oxidative stress [22, 23, 80]. A feature of long-lived bees is a high concentration of vitellogenin in the hemolymph. The highest level of vitellogenin is registered in the ovaries of queen bees, where it performs its main function in the development of oocytes [26]. Nevertheless, a high level of vitellogenin content in the tissues of queens is, apparently, one of the factors of their extreme longevity (several years) in comparison with other castes of the honeybee.

Vitellogenin is synthesized in hypopharyngeal glands of worker bees at the nurse stage and is present in the brood food [86]. The level of vitellogenin production in worker bees is much lower than that in queens [23]. However, the vitellogenin level could rise significantly as a result of its prolonged synthesis in worker bees with a low titer of juvenile hormone and insignificant use at brood withdrawal from the family [22]. Under natural conditions the honeybee subspecies *A. m. mellifera* and *A. m. carnica*, inhabiting the temperate zone, differ from subspecies of subtropical and tropical zones by the presence of temporary forms of worker bees, which accumulate vitellogenin to the levels in the queen and live for 6–10 months [79]. These facts suggest that the long-lived phenotype of the "winter bees," which allows the bee family to survive in the winter period with no restoration of the strength at the expense of the brood, appeared through developing of the ability to accumulate vitellogenin.

Honeybee vitellogenin can affect cellular and biochemical processes by modulating the signaling pathways affecting the metabolism in general [23], and thus it influences the aging process [87]. It starts a series of cascade processes acting as a factor regulating the endocrine system of insects in general through stimulation of insulin-like peptide. Vitellogenin influences the ratio of titers of juvenile hormone and 20-hydroxyecdysone [88]. The changes also concern the level of expression of the transcription factor dFOXO in the fat body [89, 90], which has a significant impact on the reproductive system of females and their resistance to oxidative stress. A group of FOXO proteins plays an important role in response to various kinds of stress and regulates a wide range of cell reactions through a change of metabolism, from the retardation of cell cycle and differentiation processes to aging and apoptosis. It defines the role of FOXO-dependent mechanisms in determination of the honeybee's life expectancy. Activation of the insulin/IGF-1-pathway leads to stopping of FOXO activator function. Under stressor activity, the insulin pathway is

inactivated and JNK and SIRT1 is induced. Dephosphorylated transcription factor FOXO moves from the cytoplasm to the nucleus, which stops cell growth and, in turn, leads to increase of resistance to oxidative stress (FOXO-regulated genes include genes encoding SOD and catalase) and life expectancy [85, 88].

The molecular bases of complex social behavior of social insects are still vague. The gene encoding vitellogenin required for the formation of insect eggs performs a number of other functions in the honeybee that are related to social labor organization. Vitellogenin protein affects the age at which a worker bee stops working inside the colony and starts foraging, when it will collect nectar or pollen, as well as its duration of life [79]. It is possible that such behavior could occur because of desynchronization of the work of genes responsible for reproduction and care for the brood. Thus parental instincts of worker bees appear before their reproduction period starts. With no brood of their own, such insects have been forced to take care of closely related members of the colony [91].

Gene regulators initially associated with reproductive function, for example, influencing the production of gonadal hormones, can be expected to play an important role in the regulation of labor organization in the honeybee [92]. Vitellogenin gene in insects, both social and nonsocial, is closely linked to female reproductive function: Protein encoded by this gene is necessary for the formation of eggs in the ovaries of females. However, this gene is also active in worker bees that do not oviposit. Moreover, its activity in worker bees has been observed to decline with age. This means that vitellogenin serves as a singular behavioral "switch": Decrease of its concentration forces a bee to leave household chores and to start foraging outside the hive at some moment of life. Thus the same gene that was originally associated with female reproductive function started to perform a number of new functions in the honeybee such as regulation of labor organization and social structure.

It has been found that in worker bees a wide range of enzymes involved in the processes of glycolysis, ATP synthesis, and generation of free radicals depends on the changes of tasks performed by the bees: from functions inside the hive to collecting of nectar or pollen [14, 93]. Evolution of the insect social castes with different reproductive potential is also associated with changes in metabolism that affect the life span [94]. From this point of view, evolutionary development of the honeybee supposes a positive connection of vitellogenesis and life span, which is not regulated only for the queen bee. This occurred because the fertility and longevity of the queen bee determines the viability and development of the colony [95]. Thereby, the honeybee is an example of how socialization in the process of evolution and changes in the scheme of reproductive potential control in the colony may be interrelated with life prolongation [94, 96–98]. This assumption is based on analysis of the interdependence of vitellogenin levels and life expectancy of the queen bee, as well as the dynamics of reproductive protein and structure of longevity in worker bees.

In addition, use of vitellogenin for stress resistance management of worker bees has been evolutionary confirmed. In worker bees vitellogenin begins to synthesize when health functions in the nest are performed prior to the function of feeding [99]. However, the level of synthesis of vitellogenin in worker bees is lower in comparison with that of the queen [100]. The protein content is depleted in the period from the performance of health functions by the working bee to larvae feeding [24]. Thus the absence of brood is a necessary but not sufficient condition for the emergence of long-living worker bees [79, 82]. Modulation of regulatory mechanisms of vitellogenin on the reproduction and maintenance of homeostasis may be recombined in queens and worker bees during the migration of the species to temperate regions [101, 102]. This recombination of functions between longevity increase and stress resistance contributed to the emergence of the phenotype of long-living worker bees, which promoted the maximum survival of the colony during the winter, when the worker bees could not be replaced because of environmental restrictions on brood raising.

20.5 WINTER GENERATION OF HONEYBEE

Before human intervention the species *A. mellifera*, having mastered West Asia, Africa, and Europe, was scattered so widely that its further evolution was in radically different natural climatic conditions. This factor led to the formation of 25–30 subspecies [103], differing in a number of features, having a specific reaction to the external environment, and often not even able to exist under artificial latitudinal displacement within the species area [104].

It is known that honeybee defensive reactions are characterized by clear interspecific differences [65, 66]. Honeybee subspecies differ in expression of antimicrobial peptide genes, the dynamics of PO activity, and hemocyte response [67, 105–107]. Interspecific differences are manifested in the reactions of the defensive systems of the honeybee to bacterial contamination, expressed in increased phagocytic reaction and an earlier activation of redox processes in *A. m. mellifera* in comparison with *A. m. caucasica* [108]. These results confirm that during the honeybee's evolution modification of adaptive properties did not concern individuals, but a colony in general as an integral biological unit [91].

A phenomenon of the winter generation of bees, which appeared (or was preserved) in the evolution in the subspecies living in conditions of pronounced change of seasons, winter to summer, is of particular interest in the issue of interspecific differentiation. This feature is characteristic of subspecies of temperate climates, such as *A. m. mellifera* and *A. m. carnica*, but is absent in the subtropical subspecies *A. m. scutellata* and others [79, 95]. It was established that worker bees can accumulate levels of vitellogenin up to 50–60% of the hemolymph protein fraction because of limited brood raising [99, 110] and live for 6–10 months [87, 111].

Winter generation is characterized by a high life span and delayed organism aging, which is associated with influence of the protein vitelloogenin and hormonal balance on the process of free radical oxidation. This feature allows the bee colony to survive the harsh long winter and to cope with many pathogens. An additional tension in the honeybee organism occurs during the long winter season in conditions of the northern part of the species habitat, for example, in Russia where the nonflight winter period lasts 5–7 months. During this time the intestines of the honeybee collect from 20 to 70 mg of undigested food residues. In this situation, the balance in the system ROS-antioxidants is especially important for the survival of the colony. First of all it relates to neutralizing putrefactive processes in the intestine. Otherwise, these residues are allocated by the bee inside the hive and can lead to the outbreak of nosemosis and other diseases.

The European dark honeybee subspecies (*A. m. mellifera*) is the most evolutionarily adapted to northern conditions. Intensive studies of features of the honeybee adaptation to a long, cold winter conducted by MV Zherebkin (1979) [112] showed that life span varies between bees appearing by (before) the warm season and focused on honey gathering during the entire plant vegetative period and bees appearing in the colony by the cold season for wintering provision. These differences are associated with the larval diet. The most winter-hardy subspecies, the European dark honeybee is characterized by a relatively large body mass of individuals and a greater volume of stored reserve nutrients, as well as a significant number of high-quality food stocks in the nest for successful wintering of the colony. The latter aspect largely determines the potential ability of different strains (types) of the honeybee to produce the total volume of honey in beekeeping. In the body of winter bees the amount of unstructured water gradually decreases and pharyngeal glands, ovaries, and fat body remain developed for a long time. Thus MV Zherebkin showed that in the process of winter preparation the majority of bees in the colony acquired characteristics of physiologically young bees with well-developed pharyngeal glands that produced large amounts of protein.

Subspecific differences include the specific functioning of the antioxidant defense systems in the gut and hemolymph of the honeybee and its distinctions in adaptation to climatic factors and exposure to the pathogens [66]. Comparative analysis in the climatic conditions of the Southern Urals revealed significant differences in the character of the stress reaction of bees of native subspecies *A. m. mellifera* and subspecies *A. m. caucasica*, introduced from the Black Sea coast. *A. m. mellifera* is characterized by a lower level of redox processes in the norm, a higher reactivity of the antioxidant enzymes, glucose-6-phosphate dehydrogenase and enzymes of PO cascade under the action of pathogens, but high and stable levels of glycosaminoglycans. This level of metabolic processes is justified in these natural climatic conditions, since a higher level of protective system response is necessary in case of threatened breach of homeostasis, ensuring response adequacy. A higher metabolic rate is constantly observed in introduced honeybees, which negatively affects the viability of the individuals if there appears excessive functional stress associated with any negative factor. These and other features of the protective reactions of *A. m. mellifera* reflect the stability of the European dark honeybee to climatic factors of the northern part of the species range [66].

Experimental data showed exhibition of normal peroxidase activity in *A. m. mellifera* honeybees predominantly in brain tissues, fat body, and the thorax muscles and mainly in the tissues of the midgut and large intestine in *A. m. caucasica* bees. Catalase activity in *A. m. mellifera* is much higher in the brain and hemolymph in comparison with other organs and tissues, and in the large intestine catalase activity increases only at the end of winter (prolonged nonflight period) in order to protect the bee organism from putrefactive processes in the intestine. This feature is also functionally fair for *A. m. mellifera*. In *A. m. caucasica* catalase activity is localized mainly in the tissues of the fat body and gut, organs that provide the highest level of metabolism [108]. This creates the conditions for maximum antioxidant protection, in particular, the catalase-peroxidase system [113, 114]. The high metabolic rate that is a characteristic of the southern subspecies as a whole will contribute to the emergence of stronger pathogenetic processes during development of stress reaction and reduce viability under the natural climatic conditions of the northern part of the species area.

During wintering not only behavior and metabolic rates of the honeybee individuals but also similar characteristics of the whole colony change, which is associated with changes in both external environmental factors

and the microclimate in the nest. Increase in the temperature in the nest during the winter is accompanied by a change in the ratio of CO_2 and O_2. In winter there is a transition of bees from aerobic to predominant anaerobic respiration in conditions of a high CO_2 content.

In conditions of reduced oxygenation, when the intensity of the Krebs cycle is reduced, in the mitochondrial respiratory chain substrates supplied by AOS (GSH, TRX, ascorbate) can be oxidized. Production of the alternative substrates of oxidation by AOS is conjugated with $NADPH_2$-generating mechanisms that are resistant to hypoxia and anoxia. A high activity of MnSOD is noted in the mitochondria. In intact cells in situ significant concentration of $O_2^{\bullet-}$ is not detected and leakage of electrons cannot serve as a sufficient ground for the destructive changes in mitochondria [115, 117]. In addition, in mitochondrial cycles the main constriction factors are catalase and enzymes with functions of GPX. Still-unknown functions of mitochondrial catalase and GPX-like enzymes have been proposed. These functions can be reduced to the transformation of H_2O_2 to O_2 and the subsequent intensification of respiration and ATP synthesis. Therefore, inhibition of catalase leads to a sharp decrease in the rate of respiration and oxidative phosphorylation in the mitochondria in vitro and in vivo. Flavoproteins possessing peroxidase activity play a major role in the process of linked oxidative phosphorylation [117]. TPX may also be such a flavoprotein, exhibiting peroxidase activity. The enzyme is multifunctional: It is involved in the peroxide metabolism of oxygen, restoring TRX and ascorbic acid and formation of deoxyribonucleotides in the metabolism of selenium-containing compounds. An important characteristic of the enzyme is its high resistance to respiratory toxins, which greatly distinguishes it from catalase and GPX [118].

20.5.1 Regulation of ROS in Honeybee Intestine

Features of climatic zones potentially affect honeybee behavior, physiology, and metabolism, as well as the development of interaction between the honeybee and pathogens [119]. In this regard, the intestine of the honeybee, which is one of the key points of the honeybee's interaction with its environment, is of great interest. In the first place, the intestine is the main organ of digestion, where nutrients and toxins, as well as enteric pathogens first come. The intestine of the honeybee in many respects determines resistance to disease at the level of individuals and the colony as a whole [120, 121]. The intestine was not randomly chosen as the main object for studying of the gene expression of different nature in the search for the causes of the Colony Collapse Disorder phenomenon [122].

TLR 3, Vanin-1, Ferretin 2, and chitinase expressed in the intestine and forming the basis of innate immunity in the honeybee differ in activity in different subspecies of the honeybee and determine the features of the confrontation of pathogenic microorganisms in different climatic conditions. Tool-like receptors are involved in the recognition of viral pathogens and also activate necrosis of the intestinal tissue under inflammatory reactions [123, 124]. Vanin-1 mediates oxidative burst in the intestinal epithelium, which occurs through the activation of the Tool/cytokine signaling pathway [125, 126]. Ferretin 2 also participates in the oxidative stress reactions; in this case it sequesters the excess ferric iron, reduces the generation of H_2O_2, and decreases the effects of oxidative stress in response to the activity of such proteins as Vanin-1 [127]. Chitinases are involved in the inflammatory response in the intestine induced by cytokines [128]. In addition, insect chitinases modulate the peritrophic membrane thickness, which forms a structure that separates the undigested nutrients from the intestinal epithelial cells [129].

The functioning of the basic biochemical mechanisms of the honeybee has been found to express caste and subspecific differences. Differential expression of the proteins involved in response to pathogens proves that various subspecies of the honeybee may have different levels of susceptibility to disease. These local adaptive responses may take place in the levels of protective protein expression, which may occur because of genetic or epigenetic changes in different subspecies of the honeybee. Metabolism in the honeybee subspecies is adapted to conditions in the place of their origin, so that the same climatic conditions have different effects on the biosynthesis, stability, and activity of proteins. All this contributes to the adaptation and conservation of the ecotypes. Therefore, measures should be taken for the study and preservation of species diversity of bees to prevent the loss of this rich genetic material that is so valuable to the world's biodiversity.

20.6 CONCLUSION AND PERSPECTIVES

The development of a unique ecological niche in the evolution of the honeybee defined a special role of oxidative stress in the vitality of the species.

One of the leading factors in the progressive evolution in the number of Hymenopteran species is the development of social instincts. In eusocial species there is a close contact with grown offspring, constant care of it at all stages of development. Communication means, maintenance of the optimal conditions of microclimate in the colony, and protection against enemies and adverse conditions have been significantly improved. Within

the colony there is a morphological and functional differentiation of individuals. In the individual actions of each individual the hereditary program that activates the mechanisms of organism alteration at certain times, physiological processes, and certain types of behavior plays a major role.

In the genome of the honeybee there are relatively few genes associated with innate immunity and detoxification proteins. In the evolution of the honeybee adaptation did not change the attributes of individuals but features of the bee colony as an integral biological unit. In accordance with this, the functioning of a large part of the genome is refocused on solving problems related to ensuring eusociality. Nevertheless, the reaction of phagocytosis in the hemolymph to pathogen introduction, the effect of a toxicant or other negative environmental impact, is accompanied by oxidative burst. It is traditionally appeared in the ROS generation and changes in antioxidant enzyme activity. Oxidative stress is a universal high-performance reaction of the honeybee organism to the impact of negative factors. Functional expression of hemocyte activation is the formation of quinone intermediates of melanin, $O_2^{\bullet -}$, H_2O_2, and other ROS with high cytotoxic activity. Change in hormonal balance during the metamorphosis of the honeybee larvae and pupae and the appearance of macrophagocytes participating in processes of histolysis in the hemolymph are also accompanied by oxidative burst and induction of the molecular mechanisms to restrain it. Hormone-dependent changing of the functions performed by the working bee in the colony and control over longevity are also worthy of thorough study, including gerontology. Thus the honeybee is not only a subject economically and socially important to the human but also a very interesting model for studying of the mechanisms of memory, behavior, immunity, etc. In a complex system of protective reactions of the honeybee, oxidative stress and molecular mechanisms of its regulation demonstrate a high efficiency in solving a wide range of tasks with strictly limited resources. The honeybee genome [12] and the interpretation of its operating principles are expected to give a better understanding of the features of *A. mellifera* species.

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