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Glutathione Levels during the Mosquito Life Span with Emphasis on Senescence (41867)

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Abstract. Glutathione levels were determined in mosquitoes of all ages of the life span. Specific analyses for reduced (GSH) and oxidized (GSSG) glutathione were used and validated to ensure minimal autoxidation of GSH and conversion of these forms. Indeed GSH accounted for >97% of the total glutathione (GSH + GSSG) content in all samples. Marked changes occurred during the life span, and the highest levels of GSH and total glutathione were found during larval growth and metamorphosis ($P < 0.001$). Thereafter the levels decreased in the early adult, plateaued in the mature, and decreased 46% in the old and very old mosquito ($P < 0.001$). This aging-specific decrease was a general phenomenon, for it occurred in all body regions of both sexes. Starvation up to 3 days did not affect the GSH levels. The importance of these changes in glutathione is its relationship to the reducing and biosynthetic capacities of different life span stages. Of special interest is the senescence decrease which can lead to lower biosynthetic activity and also impaired detoxification capacity.

The high levels of glutathione in many animal and plant cells suggest it may have important biological functions. Indeed, this tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant thiol reducing agent in tissues. Glutathione participates in a variety of biosynthetic and detoxification reactions as described in a number of reviews (1–7).

Glutathione has been implicated in the growth and development of a number of organisms. Changes in levels were observed during fetal development in mouse liver (8), the postnatal growth of rat liver (9), the asexual life cycle of neurospora (10), and the embryonic development of the sea urchin (11).

A possible role of glutathione in the aging process was demonstrated by our finding that aging-specific decreases in glutathione status occurred in various tissues of the mouse. In erythrocytes marked decreased in GSH content, free thiol groups, and GSSG reductase activity were observed as a function of cell age as well as mouse age (12). Recently similar aging-specific decreases in GSH and also total glutathione concentrations were found in other, more typical tissues (13).

There has been no systematic investigation of glutathione during the growth and aging stages of a multicellular eukaryote throughout its life span. To this end we selected the mosquito as an appropriate model since it has the following advantages. First, it is a multicellular organism that resembles mammals in its biochemical composition and nutritional requirements for growth. Second, it has discrete, synchronizable life span stages of growth, maturity, and aging, which have been characterized biochemically. Third, it can be cultured axenically and aged under controlled and defined nutritional conditions. Fourth, its short life span enables the study of the entire life cycle in 6 weeks.

Our current hypothesis is that glutathione content is related to the level of biosynthetic activity and the age of the organism. Thus the objective of this investigation was to determine the GSH and GSSG levels in tissue samples at different stages of the life span that differ in biosynthetic level. The biochemical basis for this approach was the high DNA content and biosynthetic activity found in mosquito larvae and the low values in the mature and senescent adult (14, 15). A preliminary account of this work has been presented (16).

Materials and Methods. *Experimental organism and its culture.* The experimental organism was the yellow fever mosquito, *Aedes aegypti* (Louisville), which has been colonized in this laboratory for over 17 years amounting to about 250 generations. Under our standard conditions, the larval period lasts approximately 7 days, and the pupal period, 2 days.

The chronological and biological ages of adult mosquitoes were as follows: 0–5 days,

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late metamorphosis; 6–22 days, mature; 23–35 days, old; 35+ days, very old. These age periods were characterized from survival curves and from DNA and protein profiles (14) of cohort populations that were cultured and aged under our standard conditions. The median survival time for these mosquitoes was 29 ± 2 days, a value which has been reproducible for several decades.

Standard culture. Our standardized culture procedure was used to produce mosquitoes of all ages of the life span (17). The procedure is outlined as follows: Eggs stored at 29°C for less than 1 month were hatched by agitating them for several minutes in distilled water on a Vortex mixer. Almost all larvae emerged within 15 min and were transferred to pans containing distilled water with a mineral supplement corresponding to that in axenic media. An aqueous 19% (w/v) suspension of desiccated hog liver (Teklad, Madison, Wisc.) was added daily to maintain a bacterial infusion which is the principal source of nutrients for the growing larvae.

Pupae were transferred to distilled water for adult emergence. Adult mosquitoes were fed a 10% (w/v) sucrose solution on cotton pads. No blood meals were offered to the experimental groups, and thus ovarian development was prevented. An environmental temperature of 29°C was maintained throughout the life span.

Axenic culture. Mass cultures of mosquito larvae were grown axenically on a defined medium as described by Lang *et al.* (18). Both axenic and standard, nonaxenic mosquitoes have the same rates of growth, pupation, and adult emergence under these conditions.

Tissue preparation. The protocol for each daily experiment included samples from different age groups. To determine total glutathione (GSH + GSSG), homogenates (9% w/v) of cold-inactivated larvae or adult mosquitoes were prepared in ice-cold 5% (w/v) *meta*-phosphoric acid using an all-glass, Ten-Broeck homogenizer. The actual total weights of mosquitoes used varied from 90 to 300 mg. Portions of the homogenates (1.0 ml) were centrifuged at 14,000*g* for 15 min in a Lourdes LRA centrifuge with a 9RA rotor. The supernatants were then diluted with 0.1 *M* sodium phosphate/0.005 *M* EDTA, pH 7.5.

A different process was used for GSSG. Mosquitoes were homogenized in ice-cold 0.01 *M* *N*-ethylmaleimide and incubated for 5–10 min at 4°C. Then metaphosphoric acid was added to a final concentration of 5% (w/v), and the homogenate was centrifuged. Excess *N*-ethylmaleimide was removed by extracting eight times with equal volumes of ether, and residual ether was removed by bubbling air through the samples for 20–30 min.

For recovery experiments known amounts of GSH or GSSG were added to different samples before homogenization. Each sample consisted of 40–50 mosquitoes for GSH determination and 90–100 for GSSG.

Samples of head, thorax, and abdomen were obtained from cold-inactivated mosquitoes which were trisected at the head–thorax and thorax-abdomen junctions with stainless-steel needles. Pooled samples of each body region were processed as described above.

Glutathione assay. Total glutathione and GSSG were assayed by a modification of the enzymatic cycling method of Tietze (19) and Owens and Belcher (20) in which the rate of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reduction is proportional to the amount of either GSH or GSSG present. Tissue extracts (volume 25–100 μl) were added to an assay medium containing 0.5 EC unit of yeast glutathione reductase (1 unit will reduce 1.0 μmole GSSG/min at pH 7.6 at 25°C), 0.5 μmole of DTNB, and 0.1 *M* sodium phosphate/0.005 *M* EDTA buffer, pH 7.5, in a volume of 0.9 ml. This reaction mixture was preincubated for 2 min at 20–22°C to allow thiol components in the tissue extract to interact with DTNB. Then the enzymatic cycling reaction was initiated by the addition of 0.1 ml of NADPH equivalent to 0.2 μmole, and the rate of DTNB reduction was determined from the increase in A_{412} using a Zeiss PMQ II spectrophotometer. This rate was corrected for the reaction of DTNB with glutathione reductase without tissue sample. Each sample was analyzed at two or more concentrations to ensure that the initial rates were proportional to sample size for all tissues. Also, every daily assay included standard curves using known amounts of GSH and GSSG. GSH was calculated from the difference between the total glutathione and GSSG values.

Chemicals. NADPH of 98% purity, yeast glutathione reductase [NAD(P)H-GSSG glu-

tathione oxidoreductase, EC 1.6.9.2] (200 units/ml). GSH, GSSG, 5,5'-dithiobis(2-nitrobenzoic acid), and *N*-ethylmaleimide were obtained from the Sigma Chemical Company (St. Louis, Mo.). Solutions of these reagents were prepared in 0.1 *M* sodium phosphate/EDTA buffer, pH 7.5. Other chemicals were reagent grade, and double-distilled water was used.

Statistical methods. Student's *t* test was used for statistical comparisons (21). Statistical procedures were facilitated by use of a Hewlett–Packard minicomputer.

Results. *Validation of the method.* An important aspect of this investigation was the use of an analytical method specific for both GSH and GSSG. The enzymatic cycling method of Tietze (19) was selected and evaluated for mosquito samples by determining optimal assay conditions and by recovery experiments.

The measurement of glutathione was based on the initial rates and time courses of the enzymatic reduction of DTNB. For the assay of either total glutathione or GSSG in larval or adult samples, A_{412} was proportional to time for the initial 2.0 min. Thus for routine assays the initial rates were determined within the first 1.5 min. Also, several concentrations of each sample were assayed to ensure proportionality between initial rates and sample size. In addition, mixing experiments with samples of high and low levels were performed to detect the presence of any endogenous inhibitors or activators. None was found, for the results were additive.

Recovery experiments were carried out by adding known amounts of GSH or GSSG to mosquito samples before homogenization (Table I). The recoveries of GSH or GSSG were quantitative and ranged from 92 to 108% with various added amounts and with different ages of organisms. This evidence indicated that the processing and analytical methods were valid for the quantitative determination of both GSH and GSSG.

Glutathione levels. The levels of GSH, GSSG, and total glutathione during the life span of the mosquito are shown in Table II. The data are expressed per milligram of fresh tissue since our previous studies showed that weight, DNA, and protein contents are equivalent biochemical parameters of cell size and number during all stages of the life span (14, 15). Throughout the life span the GSH content was greater than 97% of the total glutathione. Thus the GSH profile was essentially identical to the total glutathione profile.

The relative GSH content during the different stages of the mosquito are presented in Fig. 1. GSH was found in the newly hatched larva and thus was present in the diapausing mosquito embryo. During larval development the GSH content increased two-fold and reached a maximum in the 3-day-old larva.

TABLE I. RECOVERY OF GLUTATHIONE ADDED TO MOSQUITO SAMPLES

Stage	Expt	GSH or GSSG (neq of GSH/sample)	GSH		GSSG	
			neq of GSH added	Recovery (%)	neq of GSH added	Recovery (%)
Larval	1	208 (GSH)	162	103	—	—
			324	99.1	—	—
	2	6.00 (GSSG)	—	—	6.48	108
					13.0	94.7
Adult	1	204 (GSH)	162	98.1	—	—
			324	93.7	—	—
	2	149 (GSH)	162	93.1	—	—
			324	102	—	—
	3	3.24 (GSSG)	—	—	6.48	92.5
			—	—	13.0	98.5

Note. Known amounts of GSH or GSSG were added to different samples before homogenization and further tissue processing. The results are expressed as percentage of added glutathione recovered compared with the calculated amounts. The methods used are described in the text.

TABLE II. GLUTATHIONE CONTENT IN THE MOSQUITO DURING ITS LIFE SPAN

Stage	Age (days)	No. of samples	Glutathione (neq of GSH/mg of tissue) GSH	GSSG	Total
Larval	0	1	0.898	0.0146	0.913
	3	4	1.68 ± 0.0368	0.0169 ± 0.00193	1.70 ± 0.0371
	5	4	1.44 ± 0.0321	0.0196 ± 0.00159	1.46 ± 0.0329
	7	4	1.26 ± 0.0394	0.0152 ± 0.00231	1.27 ± 0.0407
Pupa	1	3	1.28 ± 0.0515	0.0164 ± 0.00196	1.30 ± 0.0524
Adult	0.5	6	1.41 ± 0.0241	0.0156 ± 0.00121	1.43 ± 0.0243
	5	6	1.09 ± 0.0338	0.0178 ± 0.00275	1.11 ± 0.0355
	12	6	1.07 ± 0.0338	0.0184 ± 0.00252	1.09 ± 0.0332
	15	6	0.990 ± 0.0270	0.0126 ± 0.00103	1.01 ± 0.0329
	21	7	0.821 ± 0.0287	0.0155 ± 0.00198	0.834 ± 0.0292
	25	5	0.655 ± 0.0472	0.0176 ± 0.00165	0.681 ± 0.0450
	32	6	0.593 ± 0.0381	0.0151 ± 0.00358	0.609 ± 0.0394
	47	5	0.566 ± 0.0407	0.0171 ± 0.00554	0.583 ± 0.0435

Note. Larvae, pupae, and adults of different ages were analyzed for glutathione content by the procedures described in the text. The 0-day larvae were hatched synchronously and analyzed within 30 min. The larvae and pupae were obtained by axenic culture, and adults were obtained by standard culture. The results are expressed as the means ± SEM of the indicated number of samples. Each sample consisted of 20–30 mosquitoes. The exceptions are the GSH and GSSG values for 0-day larvae, which were obtained from only one sample of ≃22,000 mosquitoes.

Thereafter the GSH levels decreased 66% in an irregular pattern during the remaining larval, pupal, and adult stages.

These changes were analyzed statistically by planned comparisons among class means of the developmental periods (21). The highest values which occurred during larval growth and metamorphosis were different from those for the mature adult period (5–21 days) which in turn were different from those for the senescent adult (25–47 days) ($P < 0.001$).

Of special importance was the close relationship between GSH content and the biological ages of the adult mosquito as shown by the superimposed GSH and survival curves in Fig. 2. After the fifth day of adult life when maturity was attained, GSH levels decreased 46% ($P < 0.001$) and the GSH profile was

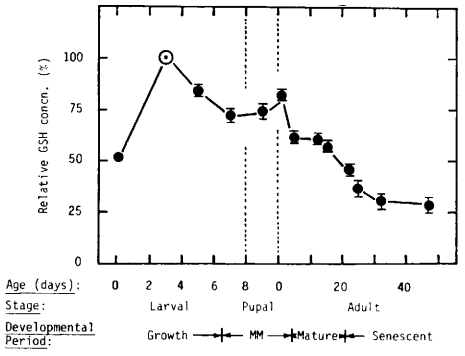

FIG. 1. Relative GSH content during the mosquito life span. Larvae, pupae, and adults of different ages were analyzed for glutathione content as described in Table II. The relative GSH content was based on the 3-day larval value, 1.68 neq GSH/mg tissue, expressed as 100%. MM denotes the metamorphosis stage.

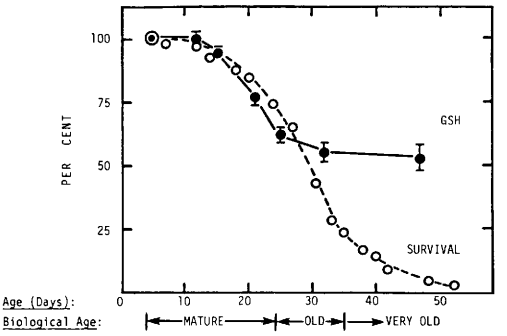

FIG. 2. The relationship of GSH content and percentage survival of the adult mosquito. Adult mosquitoes of different ages were analyzed for GSH content as described in Table II. Both the glutathione profile and the mosquito survival curve were based on their 5-day values expressed as 100%. The median survival time was 29.1 days.

TABLE III. LACK OF SEX DIFFERENCE IN GLUTATHIONE CONTENT OF MATURE AND OLD ADULT MOSQUITOES

Sex	Adult age	Weight (mg per mosquito)	Glutathione (neq of GSH)	
			Per mg	Per mosquito
Male	Mature, 12 days	1.14 ± 0.120	0.990 ± 0.0463	1.12 ± 0.0508
	Old, 27 days	1.01 ± 0.103	0.639 ± 0.0255	0.640 ± 0.0395
Female	Mature, 12 days	2.23 ± 0.0427	1.04 ± 0.0234	2.33 ± 0.0298
	Old, 33 days	2.19 ± 0.0511	0.549 ± 0.0139	1.20 ± 0.0247

Note. Adult mosquitoes raised under our standard conditions were sexed by visual examination of antennae. Biologically mature (12 days) and old (27–33 days) mosquitoes were compared. Pooled samples of 50–75 males or females were analyzed for glutathione content as described in the text. The results are expressed as the means ± SEM of three to eight samples. The GSH contents of the old groups were statistically different from those of the mature groups ($P < 0.005$).

superimposed on the survival curve until 32 days of age ($r = 0.997$). This decrease was aging specific and coincided with the rapid decrease in survivors occurring at that time. Of special note was the constant GSH content in the biologically old and very old adults (25–47 days) which suggested a critical level for survival.

GSSG contents, unlike GSH, were less than 3% of the total glutathione and were constant through the life span including the aging period.

To explore possible sex differences, adult male and female mosquitoes of mature (12 days) and old (27–33 days) biological ages were compared (Table III). The data indicated the same GSH levels in both sexes despite the twofold greater weight of the female. Also, a similar 33–49% ($P < 0.005$) aging-specific decrease in GSH content occurred in both sexes.

To determine whether the GSH changes were localized or general, different body regions of mosquito larvae and adults were analyzed. GSH was found in all body regions of both the larva and the adult with approximately 50% of the total GSH of the organism localized in the abdomen. In the 6-day-old larva the highest GSH level was found in the head (1.69 neq GSH/mg tissue), followed by the thorax (1.17 neq GSH/mg tissue) and abdomen (1.09 neq GSH/mg tissue).

In adults of all ages the highest GSH content was also in the head. However, in contrast to the larva, the content in the thorax was ~45% lower than that in the abdomen.

During adult aging GSH changes occurred in the body regions of the mosquito (Table IV). When the mature (10-day-old) and senescent (33-day-old) adults were compared, the decreases in GSH content in each body region (36–41%) were similar to that in the whole organism (38%). Also, when the GSH levels were expressed per milligram of protein or DNA, similar decreases occurred in all body regions, as well as the whole organism. Thus the decrease in GSH content during the adult

TABLE IV. AGING CHANGES IN GLUTATHIONE CONTENT IN BODY REGIONS OF THE MOSQUITO

Adult age	Region	Glutathione concentration	
		neq/mg tissue	% of mature
Young, 1 day	Head	1.86	131
	Thorax	1.00	138
	Abdomen	1.45	109
	Whole organism	1.26	128
Mature, 10 days	Head	1.42	(100)
	Thorax	0.727	(100)
	Abdomen	1.33	(100)
	Whole organism	0.986	(100)
Old, 33 days	Head	0.889	62.6
	Thorax	0.431	59.3
	Abdomen	0.846	63.6
	Whole organism	0.608	61.6

Note. Adult female mosquitoes of different ages were used as whole organisms or were trisected into head, thorax, and abdominal regions. Each sample consisted of 100–150 mosquitoes. The dissection procedure and determination of glutathione content are described in the text. The percentage glutathione contents were based on the 10-day adult concentrations as 100%.

aging period was a general phenomenon and not restricted to a specific body region.

Previous investigators have demonstrated changes in the GSH content of tissues in the rat with starvation (22, 23). To determine whether starvation was the causative factor for the aging decrease, mature and old adults were maintained on water alone for several days. At intervals mosquito samples were removed and analyzed for GSH content (Fig. 3). The content for both mature and old adults remained at the prestarvation level. Similar results were observed when GSH content was expressed per milligram of tissue or per organism. Thus the aging decrease in GSH content was not due to starvation.

Discussion. This investigation is the first systematic study of GSH, GSSG, and total glutathione contents during the life span of a multicellular, eukaryotic organism. Our results demonstrated that changes in GSH and total glutathione content occurred during the different biological stages of the mosquito, while GSSG levels were very low and unchanged.

GSH content and biosynthetic activity were correlated during the development periods for there were high GSH levels during larval growth and during metamorphosis which occurs in the pupal and the early adult stages. The maximal GSH level on the third day of larval growth coincided with maximal values of DNA, RNA, and protein contents (14) and of DNA biosynthesis (15). Also, the high GSH content in early adult life was related to high levels of protein, DNA, and electron transport enzymes occurring at that time (15, 24, 25).

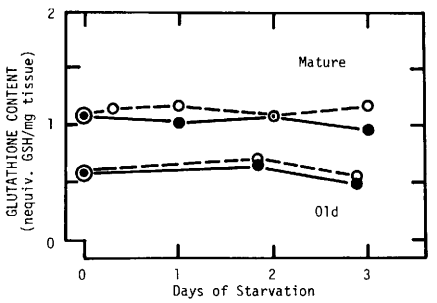

FIG. 3. Lack of starvation effect on the glutathione content of mature and old mosquitoes. Mature (13 days) and old (33 days) mosquitoes were starved by replacing their sucrose diet with water. Glutathione content was analyzed by procedures in the text. Each point is the mean of two or three samples of 15–25 mosquitoes. The exceptions are the 0.25-day-old mature, starved point and the 3-day-old, control point which consists of a single sample. ●, control; ○, starved.

GSH has been implicated in several growth processes which may explain its correlation with high biosynthetic activity. During cell division a structural function of GSH was proposed for the formation of the mitotic apparatus in sea urchin eggs but definite information is lacking (11, 26, 27). Also, the assembly and disassembly of cytoplasmic microtubules *in vitro* were related to cellular GSH content (28).

Of special importance was the GSH decrease during senescence. This change was aging specific, for it coincided with the biologically old period during which there was a marked decrease in survival. Also this GSH decrease is unusual, for the biochemical parameters of cell size and number such as weight, DNA, RNA, and protein and the free amino acid concentrations are constant in the aging mosquito (14, 29). Moreover, starvation did not affect the GSH level. Our recent evidence indicates that impaired GSH synthesis is probably the causal mechanism for the GSH decrease (29).

Equally important was our finding that the GSH content plateaus in the long-lived (<50% survivorship), senescent adult. This indicated that there may be a critical level for survival, in view of the fact that only live organisms were sampled. To prove this a longitudinal study is needed but not possible. However, it should be noted that our study is pseudo-longitudinal, since the mosquitoes were near-identical due to their relative genetic homogeneity through several hundred generations of standardized inbreeding and rigorous environmental control. Thus the determination of GSH content in mosquito samples of different ages is equivalent to repeated measurements of a single aging organism.

The decrease in GSH may be a general characteristic of many aging tissues for several reasons. First, our values were obtained initially with samples of whole organisms, and thus the decrease reflected a general aging phenomenon of most cells and not of an isolated and perhaps atypical cell type. Further our subsequent evidence showed that this GSH decrease occurred in different body regions. Second, our finding of a 37% decrease

in GSH content in the aging mosquito is similar to the decrease of 20–34% found in aging mouse and man (12, 13, 30–32).

The significance of our observation that GSH decreases with aging is exemplified by the central role GSH plays in a variety of metabolic processes. Indeed, the GSH decrease could have profound effects on the level of biosynthetic activity in cells through the regulation of enzyme activities, the synthesis of macromolecules, and the activity of the pentose phosphate cycle (6, 33–37).

Evidence for this view are our findings in the aging mosquito of marked decreases in protein and DNA synthetic rates, $NADP^+$-linked enzyme activities, and NADPH/$NADP^+$ ratios (15, 25, 38, 39). These results formed our hypothesis that a lower reducing capacity, or GSH status, occurs in aging tissue.

Another major consequence of the aging-specific decrease in GSH content is a decrease in GSH-linked detoxification capacity. Thus, old organisms could be at risk and more susceptible to toxicants. Support for this view comes from our recent findings that the toxicity of acetaminophen, a model compound for GSH conjugation reactions, was sevenfold greater in senescent compared to mature mosquitoes. Also there was a very high correlation ($r = 0.99$) between GSH content and the LD_{50} for acetaminophen (40). Further the GSH content is critical since a major function is the detoxification of peroxides produced by normal lipid metabolism and of xenobiotics via glutathione *S*-transferase, which we also found to decrease in the aging mosquito (41).

Our results indicate that GSSG levels in the mosquito are low and constant through the life span. Similar low GSSG values were found in mammalian tissues (13, 19, 42, 43). This is in contrast to the result of others who reported markedly higher concentrations (44–47). These discrepancies are most likely due to faulty methodology, for erroneously high GSSG values will be obtained due to autoxidation of GSH during sample processing and storage unless precautions are taken. In this regard Wendell (42) has pointed out the shortcomings of measuring GSSG by difference from the amount of GSH assayed before and after chemical reduction, because autoxidation of even a small proportion of GSH will appear as a large increase in GSSG.

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