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The Relationship of Catalase Activity to the Trade-Off Between Reproduction and Lifespan in the Giant Waterbug, Belostoma flumineum

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The Relationship of Catalase Activity to the Trade-Off

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BY

Matthew R. Gilg

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Abstract

Senescence is the process by which organisms age and ultimately die. Life history theory suggests that the allocation of energy into growth and reproduction is necessarily associated with a decrease in energy available for the maintenance of the soma. Many studies have shown that early or increased rates of reproduction are often correlated with a decrease in longevity, but few studies have investigated physiological correlates to this event. Catalase is an enzyme involved in the removal of oxygen free radicals implicated in damaging cellular components that contribute to senescence. A decrease in catalase activity with age could increase the organism's maintenance cost and lead to an increased rate of senescence. This study investigated the possibility that changes in catalase activity are related to the energy trade-off between reproduction and longevity in the giant waterbug, *Belostoma flumineum*. This species is a good model for this type of investigation because both males and females contribute a significant amount of parental investment.

Waterbugs were collected as fifth instar nymphs and maintained under controlled laboratory conditions. Males and females were randomly allocated to either virgin or breeder reproductive treatments. Waterbugs were assayed for catalase activity at ages of 10, 60, 100, and 150 days.

Catalase activity/ g bug was shown to increase with chronological age in male and female virgins, but not in breeders of either sex; most of this change was early in life (0-60 days). Virgin bugs also had higher catalase activity / g bug than those that were allowed to breed. This might suggest that waterbugs that breed are less protected from free radical

damage than virgins, and could help explain the shortened life span of breeders relative to virgins.

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Introduction

Senescence is an adverse change in a living organism, which is loosely correlated with the passage of time, and ultimately leads to death (Comfort, 1954). One recurrent theme in senescence research is the relationship between reproduction and the rate of aging. According to the "Principle of Allocation," organisms allot a finite amount of energy for three essential purposes; growth, maintenance and reproduction (Gadgil and Bossert, 1970). Therefore, the allocation theory suggests that if more energy is allocated into one area, less energy is necessarily available for the other two. For example, Calow (1979) stated that "if reproduction competes more successfully than other organismic processes for a limited supply of energy or other resources then both the future survival and reproductive performance of the parent are likely to be put at risk." A trade-off between reproduction and longevity has been shown in a variety of organisms, from arthropods (Loschiavo, 1968; Partridge and Farquhar, 1981; Tallamy and Denno, 1982; Partridge et al., 1987; Scheiner et al., 1989), and molluscs (Haukioja and Hakala, 1978) to lizards (Tinkle et al., 1970), and birds (Ricklefs, 1977).

Since numerous studies have shown a negative correlation between reproduction and longevity, some physiological correlates of aging should be present in relation to this trade-off. One mechanistic theory of aging that is noteworthy for its simplicity and its universality, is the free radical theory of aging. The free radical theory is based on the possibility that one factor in aging may be related to deleterious side attacks of free radicals on cell constituents (Harman, 1956). These reactions arise continuously, for the most part from oxygen in the course of normal metabolism, particularly in the respiratory

chain, phagocytosis, prostaglandin synthesis, and in the cytochrome p-450 system (Harman, 1956). Free radicals also arise from nonenzymatic reactions of oxygen with organic compounds, as well as from ionizing radiation (Harman, 1956).

The oxygen free radicals that seem to be implicated in various forms of deleterious reactions with cellular components include the superoxide anion (O_2^-) and the hydroxyl radical ($OH^{\cdot}$) (Ji et al., 1991). These radicals have been found to produce a variety of deleterious changes in cellular structures including alterations in membranes, collagen, DNA, chromosomal material, proteins and enzymes, and molecules modulating calcium levels in intracellular compartments (Lippman, 1983; Adelman et al., 1988; Harman, 1994). The adverse effects of free radical reactions have been found to be countered, at least in part, by endogenous enzymes, including glutathione peroxidase, superoxide dismutase and catalase, and by nonenzymatic means, such as tocopherols and ascorbic acid (Harman, 1994).

Catalase and superoxide dismutase (SOD) have been shown to be the critical enzymes for defense against oxygen toxicity in many organisms (Baird and Samis, 1971; He et al., 1994; Orr and Sohal, 1994; Sohal et al., 1995). SOD catalyzes the reduction of the superoxide anion to hydrogen peroxide, and catalase breaks down hydrogen peroxide to water and oxygen before it can dissociate into a pair of highly reactive hydroxyl radicals, which are believed to be the main agent of oxidative damage (Orr and Sohal, 1994). Larsen (1993), using different mutations of the nematode *Caenorhabditis elegans*, found age dependant differences in SOD and catalase activities. A recessive *age-1* mutation was found to have significantly higher SOD and catalase activities than the

controls as adults. The *age-1* mutation also increases the mean life span by 65% and increases maximum life span 110%. Larsen (1993) proposed that the higher activities of these enzymes may protect the *age-1* mutants from the deleterious effects of various free radical reactions, thereby increasing life span.

Changes in enzymatic activity have been found to correlate with senescence in several cases. Alcohol dehydrogenase, trehalase, alpha-glycerophosphate dehydrogenase, and esterase showed a decrease in activity, of varying degrees, with aging in *Drosophila melanogaster* (Burcombe, 1972). Several studies have also shown decreases in free radical scavenging enzymes, such as catalase, SOD, and glutathione peroxidase, with increasing chronological age (Reiss and Gershon, 1976; Hazelton and Lang, 1985; Sharma et al., 1993; He et al., 1994). This decrease in activity of free radical scavengers could result in less protection against free radical attacks on cellular structures, thereby increasing the organism's maintenance cost at old ages. Since energy is finite, however, much of the damage cannot be repaired and the organism will begin to show changes typical of senescence. Therefore, the activity of an organism's free radical scavenging enzymes could be a good marker of the physiological age of an organism.

A potential method to test whether oxygen free radicals affect the aging process is to look for differences in the activity of free radical scavenging enzymes when energy trade-offs involving the life span of the organism should be operating. If catalase activity truly has an effect on an organism's life span, then a treatment that increases the rate of aging and decreases the life span of the organism should be correlated with a decrease in catalase activity as well. If the free radical theory of aging is valid we should see two

effects when life span is shortened due to increased energy being put into reproduction:

1) age-related changes in catalase activity with lower catalase activity later in life when the organism is senescing.

2) lower catalase activity in individuals of a given age that have allocated more energy to reproduction, resulting in a shorter life span and a higher rate of senescence.

The giant waterbug, *Belostoma flumineum*, provides a good study system to investigate aging. The giant waterbug is an iteroparous insect, 18-24 mm in length, in which both sexes contribute a significant amount of parental investment, females in the form of egg production and males in the form of parental care of the eggs (Torre Bueno, 1906; Smith, 1976). Female waterbugs lay up to 150 eggs on the back of the male, who then aerates the eggs for 7 to 14 days, depending on the water temperature (Torre Bueno, 1906; Kruse, 1990). Waterbugs are paurometabolous insects that proceed through five nymphal instars before becoming reproductive adults about 45 to 54 days after hatching (Torre Bueno, 1906). This species is available in large numbers, has a relatively short life span, is easily maintained and will breed in captivity.

The fact that both parents contribute a significant amount of parental investment (Trivers, 1972) makes this organism especially suited to investigating energy trade-offs. In other organisms it is relatively easy to observe energy trade-offs in females since they usually have the larger parental investment in each clutch (Tallamy and Denno, 1982; Partridge et al., 1987; Fowler and Partridge, 1989; Reznik, 1992). Although the cost of sperm is generally considered small compared to that of the production of eggs, that is not always the case (Van Voorhies, 1992).Unlike most other species, male waterbugs allocate

energy not only to the production of sperm, but also to parental care (i.e., aerating, cleaning, etc.) of the eggs. Female waterbugs contribute a large amount of energy to the production of large eggs. Consequently, if the amount of energy allocated to reproduction has an effect on life span in the waterbug, it should be measurable in both sexes. Preliminary data, from a study currently being conducted in our laboratory suggests adult waterbugs have a mean life span of approximately 150-180 days. Virgin male waterbugs have a 20% longer mean life span than male waterbugs that breed and brood their eggs, and virgin females tend to live approximately 12% longer than females that breed.

Materials and Methods

Giant waterbugs were collected as last instar nymphs from three ephemeral ponds in Coles County, IL, USA in July and August, 1995 and July, 1996 using aquatic dip nets. Nymphs were transported to the laboratory in plastic coolers containing pond water and aquatic vegetation. Nymphs were separated and kept individually in 2 L plastic, cylindrical containers approximately 3/4 full of deionized water with pieces of floating plastic which served as perching sites. Nymphs were fed crickets twice a week and kept at normal summer conditions of 30 ^{0}C under 14L:10D photoperiod until emergence into adults; day of emergence was recorded and tabulated as adult age zero. Within 1 week of emergence each waterbug was sexed and individually marked by painting a number on its pronotum using white Testers brand model airplane paint and covering it with a fine coat of cyanoacrylate glue. Gender was determined by examining the genital plate; females possess two apical tufts, whereas males lack these structures (Menke, 1960).

Male and female waterbugs were randomly allocated into four age groups, each age group containing 11-15 virgins and 11-15 breeders, except age group I which contained 8 virgin males and 8 virgin females. Age groups I-IV were assayed for catalase activity at a chronological age of 8-12 days, 55-65 days, 95-105 days, and 145-155 days old respectively. Age group II male breeders were allowed to brood a single clutch. Age group III male breeders brooded two clutches, and age group IV males brooded 3 clutches of eggs. Age group II female breeders oviposited at least 25 eggs, age group III females laid at least 50 eggs, and age group IV females laid at least 100 eggs.

Male breeders were placed in groups of 8-10 in 38 L aquaria approximately 1/3

full of deionized water and supplied with plastic perching sites. Each of these aquaria also contained 15-20 gravid female waterbugs. Individual males were checked once every 24 hours for the presence of egg pads. Egg-laden males were removed from the aquaria and allowed to individually brood their clutch until hatching in a 2L plastic container previously described. Once males reached the assigned reproductive investment level they were maintained in a 12 L plastic storage container (PSC), 40.6 x 28 x 15.2 cm, approximately 3/4 full of deionized water with floating plastic perch sites and 5-6 other males. Female breeders were separated and kept with 4-5 male waterbugs in PSC described above. Once females reached their assigned reproductive investment level they were maintained in a PSC with 5-6 other females. Similarly, male and female virgins were kept in groups of 5-6 in PSC.

All waterbugs were maintained at 30 ^{0}C with 14L:10D photoperiod. Water in aquaria was changed weekly, while water in 12 L PSC and 2 L individual containers was changed twice weekly. Bugs were fed commercial crickets *ad libitum* and dead/ partially consumed crickets were removed every 24 hours.

Catalase assay:

Waterbugs were removed at their assigned age group and catalase activity determined. Individuals were weighed and homogenized in 5 ml of 67mM phosphate buffer, pH 7.0, in an ice bath. The homogenate was centrifuged at 15,000 ^{x} g for 10 minutes at 4 ^{0}C and the supernatant was used in the assay. The experimental enzyme solution was made by diluting 1 ml of supernatant with 5 ml of the Phosphate buffer. The rest of the assay followed methods described by Luck (1965). Each homogenate was

assayed in duplicate. Activity is expressed as moles of H_2O_2 changed to H_2O and O_2 / minute as calculated using the extinction coefficient of 43.6 $M^{-1}cm^{-1}$ (Bergmeyer, 1963).

Protein concentration:

The concentration of soluble protein was determined in order to ascertain specific activity of catalase. Soluble protein concentration was quantified using a Lowry Protein Assay as revised by Peterson (1977). Experimental solutions for each bug contained 20 ul of the original supernatant from the catalase assay, and were run in duplicate.

Statistical Analysis:

Catalase activity was calculated by two methods, per g body weight (bug) and per ug protein (specific activity). Detection of any differences in reproductive investment (number of eggs oviposited, number of eggs brooded, number of days spent brooding) among the three age groups of male and female breeders was conducted by a 1-way analysis of variance (ANOVA). A 3-way ANOVA with the main effects of gender, age, and reproductive investment was used to analyze differences in the physiological measurements between virgin and breeder bugs. Since no bugs bred prior to 10 days of age the 3-way ANOVA only analyzed age groups II, III, and IV. A 2-way ANOVA with the major effects of gender and age was utilized to detect differences between gender in bugs with the same reproductive treatments, i.e., male virgins vs. female virgins and male breeders vs. female breeders. A 1-way ANOVA was used in all treatments to detect any differences in catalase activity among bugs of varying chronological age. Any significant F-values (1-way ANOVA's) were further investigated using the Student Newman-Keuls' means comparison test. All analyses were conducted with an alpha value of 0.05.

Results

Reproductive Investment:

Table 1 shows the differences in reproductive investment among age groups for breeder waterbugs. Breeder males were significantly different at all age groups both in total number of eggs brooded ($F= 32.66$, $P<0.0001$) as well as total number of days spent brooding ($F=34.20$, $P<0.0001$). Similarly, breeder females were significantly different in number of eggs oviposited ($F=10.06$, $P=0.0027$), except for age group II and age group III, which did not differ significantly.

Catalase Activity/ g bug:

Both male and female virgins showed a significant age-related increase in catalase activity/ g bug ($F=29.84$, $P<0.0001$- males; $F=5.45$, $P=0.0044$ - females) (Fig. 1). Virgin males showed an increase in catalase activity/ g bug of approximately 178 %, while catalase activity / g bug increased about 72% in virgin females. The lowest catalase activity occurred in age group I for both males and females, then increased and remained relatively stable for the other three age groups. Catalase activity did not increase significantly over time, however, in waterbugs that bred ($F=2.12$, $P=0.156$ - males; $F=1.01$, $P=0.392$ - females) (Fig. 2). However, if age group I is treated as the early adult life "starting point" of catalase activity, the age-related patterns of virgins and breeders are very similar. Female breeders show a 47% increase in catalase activity / g bug, as opposed to the 72% increase female virgins demonstrate between 10 and 60 days of age. Male breeders show an increase in catalase activity / g bug of 80% between 10 and 60 days, while male virgins showed a 122% increase. A significant difference is also seen between

bugs differing in breeding status; virgins have a higher catalase activity / g bug than breeders regardless of age (F= 7.77, P= 0.0069). Catalase activity also varied between the sexes. Male waterbugs have higher catalase activity/ g bug than females as virgins (F= 5.24, P= 0.0259) and breeders (F=8.40, P=0.0075).

Catalase Activity/ ug Soluble Protein:

The specific activity of catalase in male virgin waterbugs (Fig. 3) increased significantly throughout their life (F= 12.4, P<0.0001), but the same trend was not seen in female virgins (F=0.126, P=0.944). Newly emerged male virgins (Age Group I) had significantly lower specific activity than at all other ages. Waterbugs that bred did not show age-related increases in specific activity of catalase (F=2.3, P=0.120). No significant difference in the specific activity of catalase was found between virgin waterbugs and those that had bred (F=0.071, P=0.791). Specific activity of catalase was also significantly higher in male waterbugs than in female waterbugs regardless of breeding status (F= 49.95, P<.0001- virgins; F=20.92, P=0.0001 - breeders).

Discussion

Catalase activity/ g bug tends to increase with chronological age in the giant waterbug. An age-related increase in catalase activity has not been reported in the literature to my knowledge. In most cases, catalase and other antioxidant enzymes have been shown to decrease in activity with increasing chronological age. Sharma et al. (1993) found an age-related decrease in catalase activity in the drosophilid, *Zaprionus paravittiger*. Maximum activity was observed during the time of peak reproduction. It is possible that we are seeing something similar in the giant waterbug since peak reproduction tends to be between approximately 50 and 150 days of adult age. The possibility remains that a significant decrease in catalase activity could occur at some point after 150 days. The original experimental design included assaying bugs at 200 days of age, but unfortunately the sample size was too small (due to death from senescence) to utilize in the study.

The difficulty in making comparisons with many similar studies is that the enzyme assays are generally tissue specific as opposed to a whole body homogenate, which I utilized. For example, Rao et al. (1990) showed not only a significant decrease in specific activity of catalase (34-56%), as well as SOD (26%) and glutathione peroxidase (33%), with increasing chronological age in rat liver, but also that less mRNA coding for each of the enzymes was present in the cells at later ages. This suggests that, at least in the rat liver, fewer antioxidant enzymes are being produced at later ages, causing a decrease in specific activity.

Other studies have shown differences in activity, as well as age-related differences

in activity, in various tissues. Reiss and Gershon (1976), for example, found that the specific activity of SOD decreased considerably (55%) in the liver of aging rats, but only slightly (17%) in the heart and no change was found in brain tissues. Similar results were reported in mice (Reiss and Gershon, 1976). Ji et al. (1991) found that cytosolic antioxidant enzymes, such as catalase and Cu-Zn SOD, showed decreased activity (34-56% and 26% respectively) with age in the hearts of rats, while mitochondrial Mn SOD increased in activity almost 100%. Hazelton and Lang (1985), however, found that glutathione peroxidase activity decreased with age in mouse liver (53%), kidney (35%)and heart (27%) tissues. With results seeming to vary among tissues, and even within cellular compartments, it is difficult to say how the activity of a given antioxidant enzyme is changing within an entire organism. Unless a large decrease in antioxidant enzyme activity in a specific location can be linked to the death of the organism, (e.g., lower catalase activity in the liver causes liver failure leading to death) tissue specific studies are too variable to be of much use.

Most of the aforementioned studies work with the specific activity of the enzyme in tissue samples as opposed to entire organisms. I do not feel, however, that specific activity is the best measurement of catalase activity in giant waterbugs. While the specific activity of catalase in female waterbugs was shown to be a great deal lower than males, in both virgins and breeders, the differences in activity / g waterbug were not as dramatic. Females tend to have large amounts of soluble protein that do not necessarilly relate to catalase activity, making specific activity measurements misleading. Body mass of waterbugs did not tend to fluctuate much in adulthood, consequently making the activity /

g bug measurement much more reliable than the activity / ug protein.

Virgin waterbugs (both male and female) have significantly higher catalase activity/ g bug than breeders. This decrease in activity in breeders may mean less protection from damaging free radical attacks, and increased maintenance costs. Because a considerable amount of energy is being devoted to reproduction instead of maintenance, in both male and female waterbugs, breeders would not be able to slow the rate of damage to cellular components and make repairs. Interestingly, the differences were not found to be age-related. Breeders consistently have lower catalase activity than virgins of the same age. If newly emerged adults are used as the "starting point" in both virgins and breeders, the differences in catalase activity were established by 60 days of age. This suggests that the onset of reproduction can cause a downward shift in catalase activity. These results were expected because breeder waterbugs have shorter life spans than virgins (Kruse and Gilg, unpubl.).

Few, if any, studies have investigated changes in the activities of antioxidant enzymes based on energy trade-offs. Dietary restriction has been shown to increase life span in a variety of organisms including rats (Duffy, et al., 1989; Rao et al., 1990) and Daphnia (Ingle et al., 1937), in the same way a decrease in reproductive investment can increase life span. Rao et al. (1990) demonstrated that specific activities of catalase, SOD, and glutathione peroxidase differ in the livers of diet restricted rats and rats fed *ad libitum.* Diet restricted rats had higher activities in all three enzymes at almost every age, and the rate of decrease in activity with increasing chronological age was slowed. The maximum differences in enzymatic activity between the two treatments were more substantial than

the differences found in the waterbug. Catalase activity was 64% higher in diet restricted rats than in rats fed *ad libitum*, while SOD activity was 38% higher in diet restricted rats.

He et al. (1994) utilized strains of senescence prone and senescence resistant mice to analyze differences in SOD and glutathione peroxidase activities. The activity of both enzymes decreased with increasing chronological age, but the differences between treatments were fairly small. At the latest chronological age SOD activity was only 14% higher in senescence resistant mice than in senescence prone mice. Glutathione peroxidase activity was only 3% higher in senescence resistant mice. These differences in activity do not sufficiently explain the 33% longer life span of the of the senescence resistant mice.

Orr and Sohal (1994) inserted extra copies of the genes coding for SOD and catalase into groups of *Drosophila melanogaster* resulting in a simultaneous 73% increase in catalase activity and a 26% increase in SOD activity. This manipulation increased mean life span up to 34% and maximum life span by about 30%. This study provides some of the best evidence that substantial increases in the activity of antioxidant enzymes can result in increased life spans.

The magnitude of the change in catalase activity/ g bug varies with sex in the giant waterbug. The maximum difference in catalase activity/ g bug between female virgins and female breeders is approximately 45% at 150 days of age. The maximum difference is only 24% in male waterbugs at 60 days of age. It is possible that these differences in magnitude between males and females are representative of the amount of energy each gender is allocating to reproduction. The production of eggs in females could be more costly than the act of brooding eggs is for males, resulting in less energy being available to

maintain catalase activity at its peak levels. An interesting anomaly also occurs at 150 days, when breeder males actually have a higher activity than virgin males. This could simply be due to small sample sizes in both treatments, but breeders having higher catalase activity than virgins later in life is not congruent with the idea that catalase activity plays a major role in the rate of aging in the giant waterbug.

Catalase activity/ g bug tended to increase early in the adult life of the waterbug, but remained relatively stable afterward. This lack of change later in life does not make catalase a very suitable physiological marker of aging in the giant waterbug. The fact that virgin waterbugs showed age-related increases in catalase activity suggests better defense against free radical damage later in life. Breeder waterbugs showed no significant increase in catalase activity with age, but enzymatic activity did not decline either. If free radicals are indeed involved in the aging process of the giant waterbug, it does not seem to be the result of a reduction in defense at later ages. It is possible, however, that free radicals play some role in the aging process in waterbugs, because breeders had lower catalase activity/ g bug than virgins of the same age. This difference may result in less defense against damaging free radical attacks, thereby increasing the organisms maintenance cost and resulting in a faster rate of senescence. The decrease in catalase activity with only one reproductive bout is also intriguing. This suggests that reproduction could potentially incur physiological costs almost immediately. Studies similar to this in other organisms would be useful in discerning the role free radicals, and free radical defense play in shortened life spans due to energy trade-offs.

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Table 1: Body weight and reproductive investment among breeder and virgin waterbugs. Wet weight of bug, total number of eggs and total number of days males spent brooding eggs are given as means with one standard error in parentheses. Superscripts denote significant differences.

Sex/ Age (days)	n	Wet Weight (g)	Total # Eggs	Total # Days Brooding
b. male/ 55-65	7	0.205 (0.006)	84.1 (7.7)^{a}	7.7 (0.6)^{a}
b. male 95-105	7	0.226 (0.008)	192.6 (18.7)^{b}	12.0 (0.8)^{b}
b. male 145-155	3	0.255 (0.006)	267.7 (9.6)^{c}	19.0 (1.5)^{c}
b. female/ 55-65	4	0.278 (0.010)	53.0 (11.9)^{a}	NA
b. female/ 95-105	6	0.278 (0.010)	80.2 (13.8)^{a}	NA
b. female/ 145-155	5	0.288 (0.027)	170.4 (26.5)^{b}	NA
v. male / 8-12	8	0.220 (0.006)	NA	NA
v. male / 55-65	9	0.224 (0.007)	NA	NA
v. male / 95-105	9	0.227 (0.007)	NA	NA
v. male / 145-155	4	0.234 (0.006)	NA	NA
v. female/ 8-12	8	0.247 (0.010)	NA	NA
v. female/ 55-65	10	0.289 (0.012)	NA	NA
v. female/ 95-105	10	0.269 (0.013)	NA	NA
v. female/ 145-155	4	0.292 (0.012)	NA	NA

b.= breeder
v.= virgin

Fig. 1: Catalase activity / g waterbug, described as moles of H_2O_2 converted to H_2O and O_2 / minute, for male and female virgins at all age groups. Each point represents the mean activity ($\pm$ 1 SE). Significant differences exist between sexes and between individuals in age group I relative to other age groups.

Fig. 1

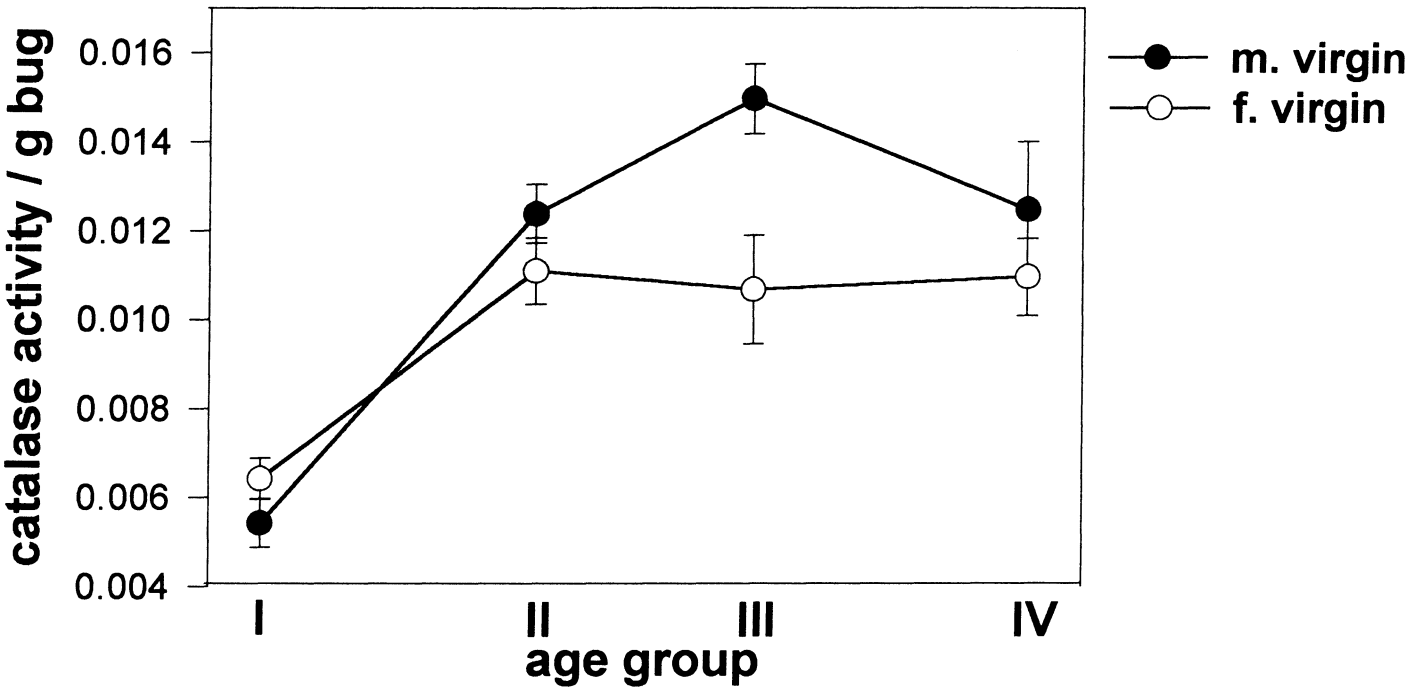

catalase activity / g bug
0.016
0.014
0.012
0.010
0.008
0.006
0.004
I
II
III
IV
age group
m. virgin
f. virgin

Fig. 2: Catalase activity / g waterbug, described as moles of H_2O_2 converted to H_2O and O_2 per minute, for male and female breeders at all age groups. Each point signifies the mean activity ($\pm$ 1 SE). Significant differences exist between sexes; there is also a significant interaction between male and female breeders of varying chronological ages.

Fig. 2

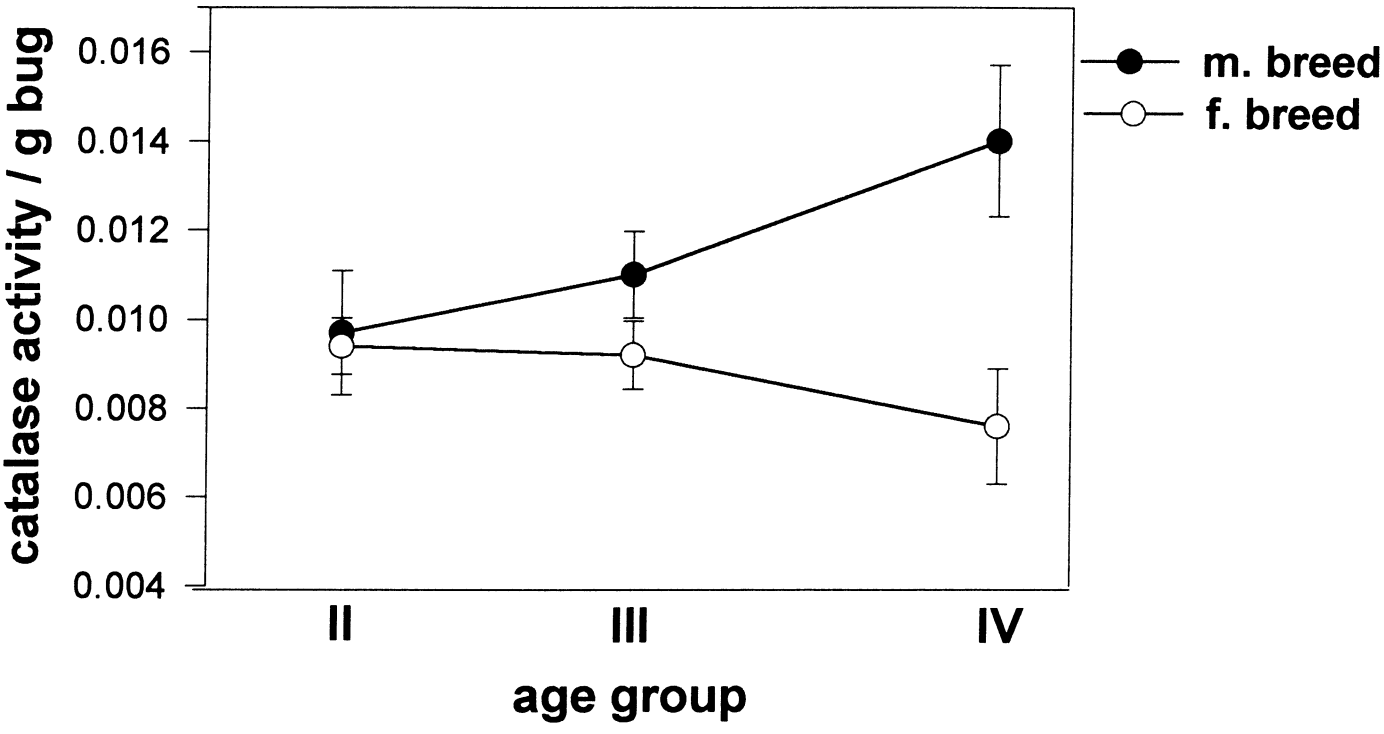
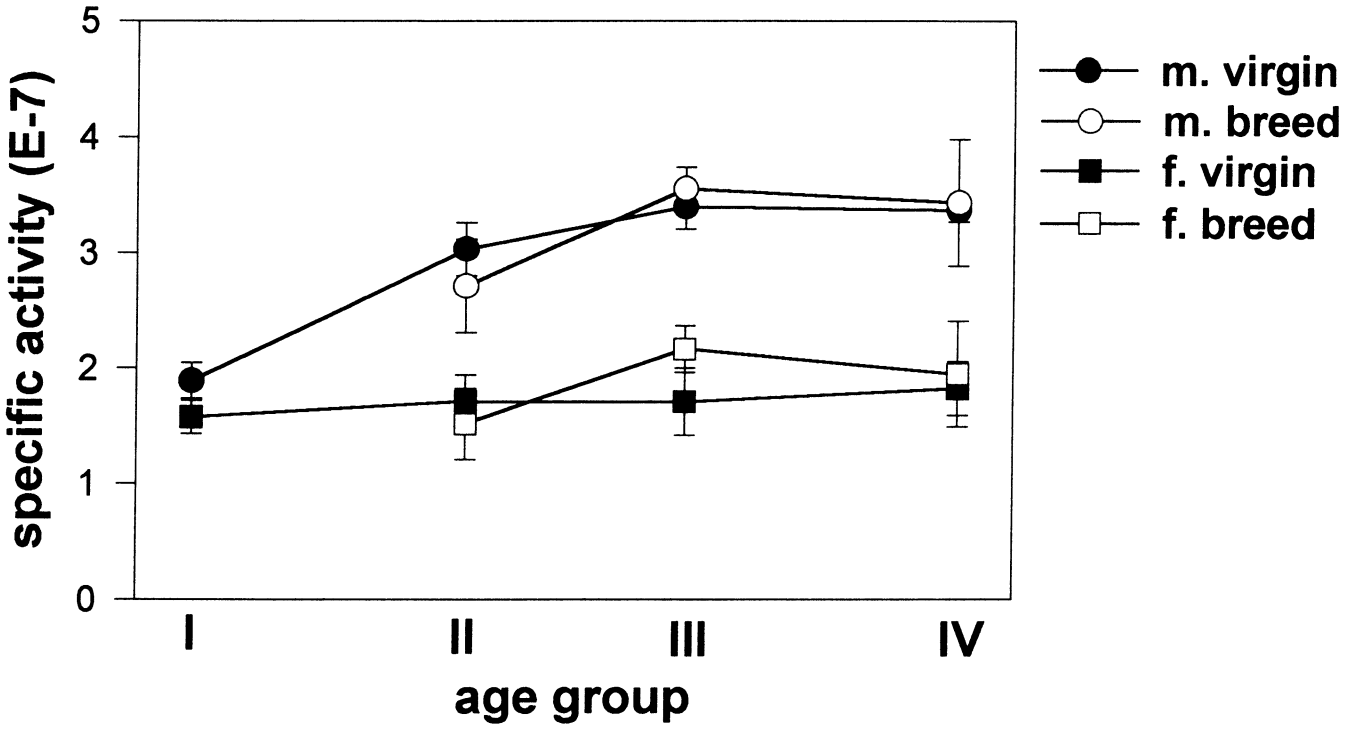

catalase activity / g bug
0.016
0.014
0.012
0.010
0.008
0.006
0.004
II
III
IV
age group
m. breed
f. breed

Fig. 3: The specific activity ($mol*min^{-1}*ug\ prot^{-1}$) of catalase for all age groups of male and female virgin and breeder waterbugs. Each point is the mean specific activity ($\pm$ 1 SE). Significant differences exist between the sexes, and with increasing chronological age in male virgins. A significant interaction is also present between male and female virgins.

Fig. 3


specific activity (E-7)
5
4
3
2
1
0
I
II
III
IV
age group
m. virgin
m. breed
f. virgin
f. breed