Title:

Evaluation of larval quality of viviparous scorpionfish *Sebastiscus marmoratus*

Running Title:

Larval quality of scorpionfish

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Abstract:

We aimed to develop an acute test for larval quality in the viviparous scorpionfish (*Sebastiscus marmoratus*). Rearing experiments until day 13 post parturition were conducted to investigate the survival of larvae for 13 different batches, and tolerance to starvation of larvae was examined and expressed by the survival activity index (SAI). We also observed the morphological characters, enzyme activity, and swimming behavior of larvae on day 0 and 1, followed by the correlation analysis between SAI. Larvae with high SAI (≥26) showed significantly higher survival on day 13 than larvae with low SAI, which confirmed that SAI is a reliable index that can be used to evaluate larval quality, similar to the former findings. The esterase activity (r=-0.713, P<0.01), swim frequency (r=-0.735, P<0.01) and swimming speed (r=-0.588, P<0.05) of larvae on day 0 were significantly and negatively correlated with SAI. We conclude that enzyme activity and behavioral characters of larvae just after parturition can be a real-time index for evaluating the larval quality of this species.

Key Words: behavior, enzyme activity, larval quality, survival, SAI

INTRODUCTION

Varying egg and larval quality leads to fluctuation of harvesting in the seedling production of marine fishes. Therefore, much effort has been made to develop evaluation criteria for marine egg and larval quality.[1,2] Egg quality is defined as the egg's potential to produce viable fry, [1] and an assessment of cell symmetry at early stages of cleavage is an example of indicators of egg quality in several marine fishes.[1-3] However, these morphological assessments of egg quality cannot be applied to a viviparous species such as scorpionfish *Sebastiscus marmoratus* which is a commercially valuable species in Japan. For the assessment of a larval quality in *S. marmoratus*, Shimma and Tsujigado [4] demonstrated that the survival activity index (SAI), which is expressed as a function of tolerance to starvation of larvae, was positively correlated to the survival of larvae. SAI was thus defined as an index for larval quality of this species. Moreover, SAI had been found as an effective index for larval quality in oviparous fishes, such as yellowtail *Seriola quinqueradiata* [5] and striped jack *Pseudocaranx dentex*. [6] Since then, SAI has been widely used as reference data determining larval quality both in experimental studies [7] and in public hatcheries in Japan.

Although SAI is an effective indicator of larval quality of *S. marmoratus*, it requires a considerable amount of time (approximately 10 days) to measure. Seedling production is carried out parallel to the SAI measurement, which is costly both for monitory considerations and hatchery time. Therefore, in order to streamline hatchery procedures some biological parameters of newly released larvae of this species, which have a significant correlation with SAI and/or survival, were examined and compared to SAI. Physiological and behavioral attributes may have significant effects on the viability and activity of fish larvae. Thus, we hypothesized that enzyme activities and behavioral characteristics will have a correlation with SAI and survival of *S. marmoratus* and aimed to provide basic information for the establishment of a real-time assessment of the larval quality.

MATERIALS AND METHODS

Materials

The broodstock of scorpionfish (body weight 250-400 g) were collected from coastal area of Nomozaki, Nagasaki Prefecture in 1998 and were held in the net cage at Nagasaki Prefectural Institute of Fisheries, Nagasaki, Japan. They were fed dry pellets until satiation 3 times a week. From November 1999 to April 2000, females were checked daily whether they had largely distended abdomen and larvae were observed in genital pore, and then females regarded as just prior to parturition were transferred individually into the 200 l black polyethylene tank with mild aeration. Water temperature was kept at 16.0-16.5 °C and light condition was natural. The timing of parturition of *S. marmoratus* varies between 17:00 and 22:00. [8] Therefore, we defined the next morning after parturition as day 0, when we could see the released larvae at 8:00, for the convenience of experimental settings. A total of 13 batches of larvae (about 40-50×10^3 larvae per female) were obtained from 14 December 1999 to 10 April 2000 and were used for the following experiments.

Rearing experiment and SAI

Rearing experiment was conducted to determine the larval survival. Ten thousand of larvae of day 0 from each batch were transferred into a 1 kL conical tank. Water temperature was kept at 16.0-16.5 °C, water exchange rate was 100 %/day and light condition was natural throughout the rearing period. Immediately after the transfer of larvae into the rearing tank (8:00), S-type of *Brachionus plicatilis* complex was fed. Rotifers were cultured with HUFA enriched *Chlorella vulgaris* (Super *Chlorella* V12, *Chlorella* Industry Co. Ltd., Fukuoka, Japan). In the rearing tank, rotifer density and green water density (Super *Chlorella* V12) were maintained at 5 rotifers/mL and at a density of 5×10^5 cells/mL, respectively, twice daily

(7:00 and 13:00). On day 0 and 1, 20 larvae were randomly sampled from the rearing tank and were anaesthetized with MS222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., SL, U.S.A.). Then, feeding activity was investigated under a dissecting microscope by examining the feeding incidence (%; dividing the number of larvae with rotifers in their guts by number of observed larvae) and by counting numbers of rotifers in each larval gut. After 2 weeks rearing until day 13, the number of survivor was counted and survival rate was calculated for each batch.

Starvation tolerance test was conducted for each batch following the method of Shimma and Tsujigado.[4] Three glass beakers containing 500 mL of sand-filtered seawater were prepared and 30 larvae of day 0 were gently introduced to each beaker. These beakers were kept in an incubator at 16.5 °C and total dark condition. Dead larvae were counted and removed with 170 mL of seawater by glass pipette and 170 mL of fresh seawater was added once daily. This procedure was repeated until all fish died. From the number of surviving larvae and survival duration (days), the survival activity index (SAI)[4] was calculated from the following equation:

$$\mathrm{SAI} = \frac{1}{N}\sum_{i=1}^{k}(N - h_i)\times i\,,$$

where N is the total number of supplied larvae, h_i is the cumulative mortality by the day i, and k is the number of days elapsed until all larvae died due to starvation. Average SAI was calculated for each batch and was used for further analysis.

Morphological, physiological and behavioral measurements of larvae

Two thousand of larvae of day 0 from each batch were transferred into a 200 L transparent polycarbonate tank with mild aeration. Fish were kept until day 1 at 16.5 °C, water exchange rate was 100 %/day, and with natural light condition.

Morphological measurements were conducted using 20 fish sampled at 10:00 of day

0 and 1, respectively. Standard length (SL, equivalent as notochord length) and diameter of oil globule for long and short axis was measured using a profile projector (Nikon, Tokyo, Japan) after anaesthetizing with MS222. A volumetric formula for an ellipsoidal solid was used for calculating oil globule volume (OGV) and oil globule consumption rate ($OGC(\%) = \frac{OGV_{\text{day0}} - OGV_{\text{day1}}}{OGV_{\text{day0}}} \times 100$). Average SL, OGV and OGC for each batch were used for further analysis.

Enzyme activity was measured for larvae on day 0 and 1 from the same tank as the morphological measurements, with slight modification after Janssen *et al.*[9] and Araujo *et al.*[10] Thirty larvae were sampled and anaesthetized with MS222 on 10:00 of day 0 and 1, respectively. Five fish were stored in a 1.5 ml microcentrifuge tube (6 groups/day/batch) at –80°C until analysis. From our preliminary observation, we could detect the activity of esterase and alkaline phosphatase from larvae. The enzymes used as substrates for esterase and alkaline phosphatase were 1.88 mM carboxyfluorescein diacetate, acetoxymethyl ester (Molecular Probes Inc., OR, U.S.A.) and 1.78 mM fluorescein diphosphate (Molecular Probes Inc., OR, U.S.A.), respectively. The sample was homogenized with 1.5 mL of seawater and then transferred into a new microcentrifuge tube, then 1.3 μl of enzyme substrate was added, and the sample was incubated at 37°C in the dark for 15 min. After incubation, the reaction was stopped by adding 20 μl of 0.5 M Sodium Lauryl Sulfate (Wako, Osaka, Japan). The tubes were centrifuged at 9000 rpm for 5 min. A volume of 1.0 mL of the supernatant was subjected to fluorescence measurements (excitation and emission wavelengths: 492 nm and 517 nm) using fluorometer (TD-700, Turner Designs, CA, U.S.A.). For the calibration of fluorescence, fluorescein (Molecular Probes Inc., OR, U.S.A.) was used for both esterase and alkaline phosphatase. Enzyme activity was defined as the amount of hydrolysed substrate per time unit (Unit, hydrolysed substrate/min) per fish with modification of Ueberschär.[11] Average value for three groups of each enzyme activity was calculated.

Behavioral observations were made at 13:00 of day 0 and 1 for each batch. About 10 fish were transferred to the observation container, which is an acrylic tank (length 7.5 × width 10.0 × height 6.0 cm) with a depth of 4 cm of seawater at 16.5°C. Illumination was kept at 1000 lx using fiber light. Fish were acclimated for 10 min prior to observation and behavior was video-recorded for 5 min from above using a digital video camera (TRV 50, Sony Corp., Japan). This observation was repeated 3 times for one age group. According to Rabe and Brown [12] and Puvanendran and Brown, [13] forward movement of the larva through water column resulting from undulations of the caudal region was defined as 'swim', and then the frequency of 'swim' (swim frequency) was counted for 3 randomly selected larvae for 1 min in the video record. Swimming distance of these 3 fish for 1 min was also measured on the video monitor and then expressed as swimming speed (mm/min). Swim frequency and swimming speed of 9 individuals was pooled for each age group of the same batch and then average value was calculated.

Statistical analysis

For the data obtained from 13 different batches, linear regression analysis was performed to examine the correlation among survival on day 13, SAI, feeding activity, morphological characters on day 0 and 1 (SL, OGV, OGC), enzyme activity on day 0 and 1, and behavioral characteristics on day 0 and 1 (swim frequency and swimming speed). Student's *t*-test was applied between 2 groups comparison. *P* value of 0.05 or less was regarded as significant for all tests.

RESULTS

Survival, SAI and morphological characters

Rearing experiment showed that survivals of scorpionfish larvae on day 13 varied

from 0.0 to 98.9 % throughout the experimental period (Fig. 1). SAI also varied between 8.8 and 41.8 (Fig. 1). Although it was not significant, positive relationship was observed between SAI and survival (Fig. 2). Moreover, distinct threshold of SAI determining the larval survival was found; the survival of larvae with SAI less than 26 was significantly lower than that of larvae with SAI more than 26 (Table 1).

SL of the larvae on day 0 and 1 varied from 3.6 to 4.1 mm, and a positive relationship was found between the date of parturition and SL (Fig. 1). However, there was no significant correlation between SL and larval survival on day 13, or SL and SAI. Feeding incidence and the number of rotifers in larval gut on day 0 and 1 varied from 0 to 100 % and from 0.4 to 26.3, respectively. OGV on day 0 was between 1.8 and 20.9 mm^3 and OGC varied from 0.1 to 91.4 %, respectively. No significant correlation was detected among larval survival on day 13, SAI, feeding activity, OGV and OGC. Even when feeding activity, SL and OGV was classified into 2 groups by the threshold of SAI (26), these values were not different significantly (Table 1).

Enzyme activity

Esterase activity of larvae on day 0 ranged from 0.0015 to 0.0071 Unit/fish, and there was a significant negative correlation between SAI (Fig. 3). Esterase activity of larvae on day 1 (0.0025-0.0074 Unit/fish) also showed a significant negative correlation between SAI (n=13, r=-2.234, P<0.05). On the contrary, alkaline phosphatase activity of larvae on day 0 (17.1-51.2 Unit/fish) and 1 (20.6-59.2 Unit/fish) did not show any correlation between SAI. When enzyme activity of larvae on day 0 was classified into high and low SAI (Table 1), esterase activity of larvae with high SAI (≥26) was significantly lower than that of larvae with low SAI, whereas no difference was found in alkaline phosphatase activity.

Behavior

Significantly negative correlation was detected between SAI and swim frequency, and between SAI and swimming speed, both on day 0 and day 1, respectively (Fig. 3). Swim frequency (/min) of larvae on day 0 was from 7.9 to 26.4, and that of larvae on day 1 was from 11.8 to 32.1. Swimming speed also varied between 38.6 and 116.0 mm/min on day 0, and between 65.6 and 116.8 mm/min, respectively. Larvae from low SAI batch showed significantly higher swim frequency and swimming speed than those from high SAI batch (Table 1).

DISCUSSION

First, we compared the relationships among early survival from the rearing experiment until day 13 after parturition, SAI values, and morphological characters of newly released larvae in the viviparous scorpionfish *Sebastiscus marmoratus*, using different batches covering the whole breeding season of this species.[14] Early survival of larvae and SAI had a trend of positive relationship. Furthermore, larvae from the batch with high SAI (≥26) showed significantly higher survival than those from the batch with low SAI, indicating that there is a distinct threshold of SAI determining higher survival of larvae. Similarly, Mushiake and Sekiya[6] reported that significantly higher production of juveniles were obtained from the batch with high SAI (>6) and the positive relationship between SAI and early survival (10 days after hatching) was found in yellowtail.[5] On the other hand, we did not find any correlation between survival and morphological characters of newly released larvae (SL, OGV and OGC), or between SAI and those morphological characters, indicating that morphological characters are not applicable for the evaluation of larval quality in the scorpionfish. Synthesizing these findings and the former findings,[4] we could confirm that SAI is reliable index that can be used to evaluate larval quality of *S. marmoratus*.

The negative relationship between SAI and esterase activity was found in newly released larvae in scorpionfish. Generally, esterase and alkaline phosphatase are involved in the digestion of the nutrient components such as glycerol esters of fatty acids, lipid, glucose, calcium, and inorganic phosphates. [15] Trypsin level, which is also an important digestive enzyme in fish larvae, had been used to asses the nutritional conditions of both wild and reared fishes such as herring *Clupea harengus*, turbot *Scophthalmus maximus* and Atlantic cod *Gadus morhua*. [11] In these 3 species, larvae in well fed condition had higher tryptic activity than starved larvae. [11] Our result was reversed with this finding presumably due to the difference in experimental conditions, where we compared enzyme activity in absence of live feed. The esterase activity of larval scorpionfish in this study may reflect the metabolic conditions of energy utilization of oil globule and muscle. In rotifers, esterase activity had been established as an index for the diagnosis of culture conditions by Araujo *et al*. [16,17] They found that high esterase activity was observed when rotifers were stressed with such as increasing free ammonia and water viscosity. Our result indicates that the measurement of esterase activity is applicable to assess the larval quality as well as rotifers. Also, trypsin activity can be a candidate for the assessment of larval quality, although we could not measure trypsin activity in *S. marmoratus* due to technical problems.

In yellowtail flounder *Pleuronectes ferrugineus* [12] and Atlantic cod, [13] larvae with high feeding incidence and swim frequency showed higher survival and growth. However, this was not the case in scorpionfish, because no correlation was found between survival and feeding activity. In this study, swim frequency and swimming speed of newly born scorpionfish larvae were significantly and negatively correlated with SAI, and larvae from batches with low SAI had significantly higher behavioral activity. Since these measurements were conducted for larvae within a controlled static environment, these behavioral characteristics should have reflected the larval condition in the absence of live feed stimuli.

Considering the result from esterase activity, behaviorally active larvae presumably waste energy more than food intake, and these findings imply that newly born larvae with high esterase and behavioral activity had some imbalance in metabolism.

We realize that RNA/DNA ratio is an effective index for fish quality, [18] and this ratio is used to evaluate the quality of chum salmon *Oncorhynchus keta* juveniles for release, [18] and to estimate nutritional conditions of Japanese sardine *Sardinops melanostictus* [19] and Japanese anchovy *Engraulis japonicus* [20] larvae in wild conditions. Even though RNA/DNA ratio is an effective biomarker for larval quality, the esterase activity and behavioral observation in this study are advantageous in terms of cost and time because these observations can be conducted with simple method and done within 1 hour. We conclude that enzyme activity and behavioral characters of larvae just after parturition can be a real-time index for evaluating the larval quality of this species.

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Table 1. Comparison of early survival, feeding activity, morphological measurements, enzyme activity and behavior of newly released larvae (day 0) of scorpionfish between the batches with low (8.8-21.5) and high (26.0-41.8) SAI.[4] Data are represented as average ± standard deviation, and asterisks indicate significant difference by *t*-test (* $P<0.05$, ** $P<0.01$), respectively.

SAI	8.8 ~ 21.5 (n=4)	26.0 ~ 41.8 (n=9)	*t*-value
Survival (%) on day 13	0.8 ± 1.7	43.1 ± 36.3	2.27 *
SL (mm)	3.9 ± 0.1	3.8 ± 0.2	1.46
Feeding incidence (%)	61.3 ± 12.5	57.2 ± 13.5	0.51
Number of rotifers in larval gut	3.1 ± 0.7	2.7 ± 1.6	0.49
OGV (mm^3)	0.011 ± 0.006	0.014 ± 0.003	1.20
OGC (%) between day 0 and 1	46.4 ± 31.6	30.9 ± 18.6	1.13
Esterase activity (Unit/fish)	0.006 ± 0.001	0.003 ± 0.002	2.88 *
Alkaline phosphatase activity (Unit/fish)	36.30 ± 11.14	26.50 ± 8.36	1.77
Swimming speed (mm/min)	103.0 ± 9.6	68.5 ± 15.1	4.16 **
Swim[12,13] frequency (/min)	23.9 ± 2.0	14.1 ± 3.6	5.07 **

Figure Legends

Fig. 1

Differences in survival until day 13 post parturition, SAI (top figure), and standard length (day 0 and 1; bottom figure) of larvae in viviparous scorpionfish *Sebastiscus marmoratus* by parturition date. SAI (Survival Activity Index) was tested to examine the tolerance of newly released larvae to starvation following Shimma and Tsujigado (also see text). [4]

Fig. 2

Relationship between SAI and survival until day 13 post parturition of scorpionfish (*Sebastiscus marmoratus*) larvae. SAI (Survival Activity Index) was tested to examine the tolerance of newly released larvae to starvation following Shimma and Tsujigado (also see text). [4]

Fig. 3

Relationship between SAI and esterase activity (top figure), swimming speed and swim frequency (bottom figure) of newly released scorpionfish (*Sebastiscus marmoratus*) larvae. SAI (Survival Activity Index) was tested to examine the tolerance of newly released larvae to starvation following Shimma and Tsujigado (also see text). [4] 'Swim' was defined as forward movement of the larva through water column resulting from undulations of the caudal region after Rabe and Brown [12] and Puvanendran and Brown. [13]

Fig.1. Matsuo *et al*. (2006)

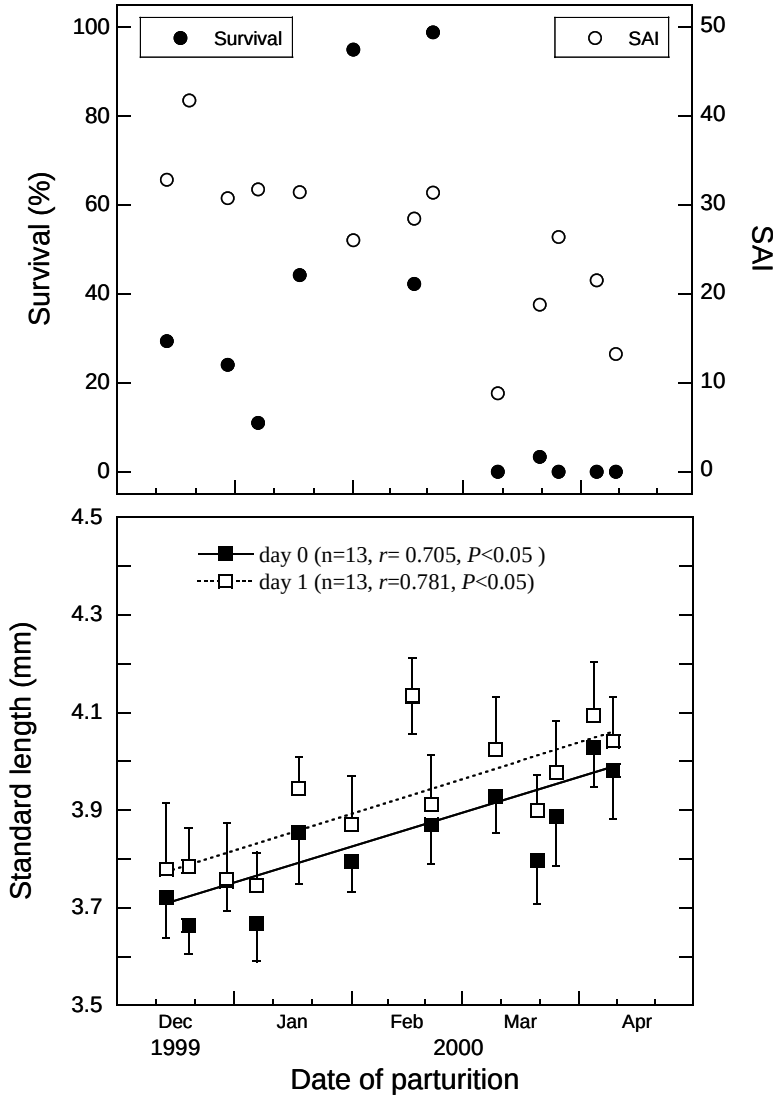

Fig.2. Matsuo *et al.* (2006)

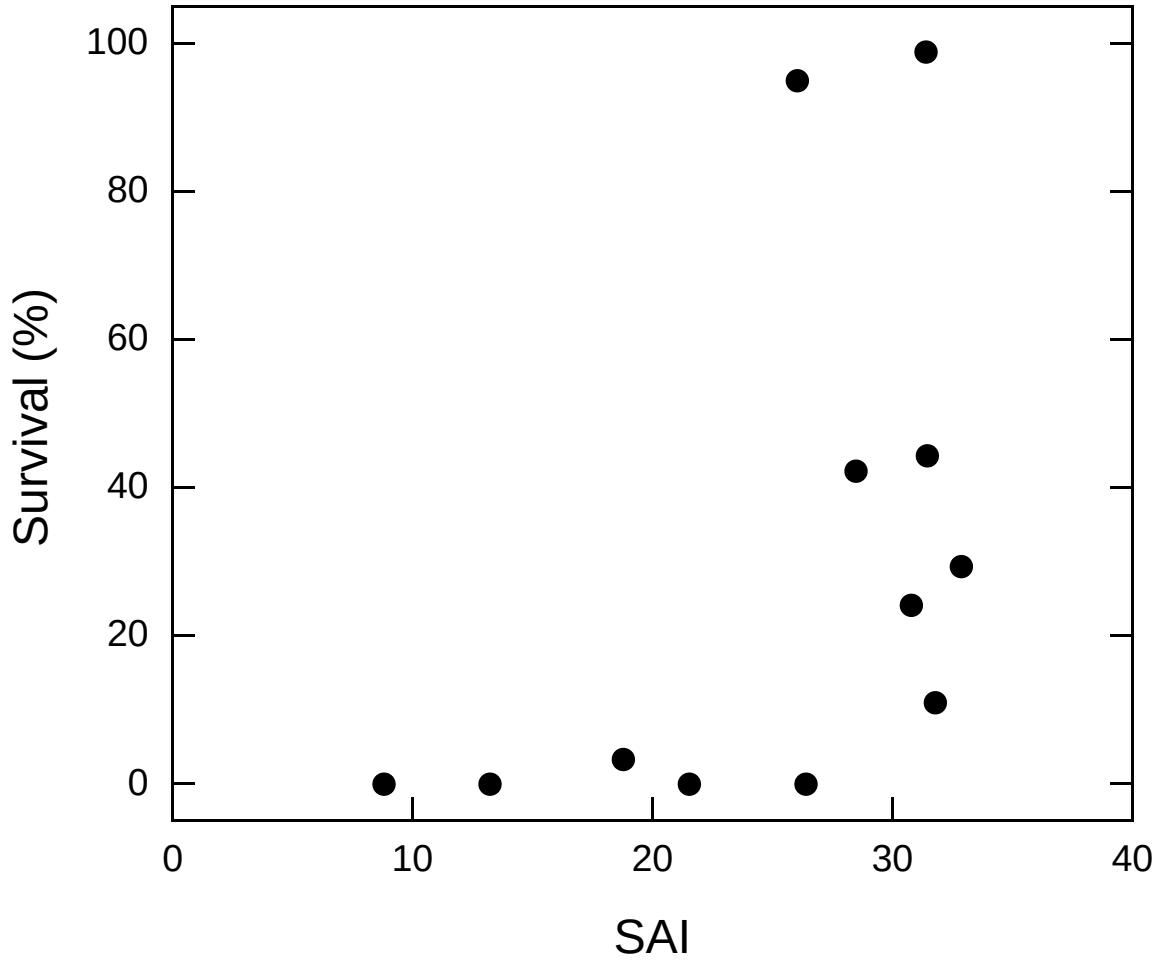

Fig.3. Matsuo *et al.* (2006)