

**Diving Behavior in *Anopheles gambiae* (Diptera: Culicidae): Avoidance of a Predacious  
Wolf Spider (Araneae: Lycosidae) in Relation to Life Stage and Water Depth**

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**ABSTRACT** It has been suggested that mosquito larvae and pupae dive to avoid predators. We tested this predator-avoidance hypothesis using immature *Anopheles gambiae* Giles and the wolf spider *Pardosa messingerae* (Stand). Because previous studies have suggested that wolf spiders are poor predators of immature mosquitoes, we first examined the predatory ability of the wolf spider and found that the spider was effective at capturing all stages of larvae and pupae. The number of mosquitoes missing from experimental cups containing deep water increased with the age of mosquitoes, with the exception of pupae. In contrast, this trend was not observed in shallow water. In particular, the number of missing mosquitoes was significantly lower in deep water during the second instar. When the effect of cannibalism was excluded by subtracting the number of missing mosquitoes for the treatment without spiders from those with spiders, the cannibalism corrected mortality was significantly lower in deep water during the second instar. The duration of diving by larvae and pupae decreased with age. With the exception of first instar, the number of diving mosquitoes also decreased with age. We postulate that this diving behavior allows *An. gambiae* to escape predation by wolf spiders, which supports the predator-avoidance hypothesis. This study indicates some important implications for vector control.

**KEY WORDS** Diving, *An. gambiae*, wolf spider, predator avoidance, malaria

## Introduction

Predator-avoidance behavior may often be constrained by cost-benefit tradeoff. Although the behavior incurs a cost (*e.g.*, energy or time required to avoid predators), this cost must be minimized to improve survival (benefit). Mosquito larvae and pupae dive when their habitat is mechanically disturbed (Gilles and Meillon 1968, Clements 1999), and even a shadow passing over the surface can elicit diving behavior in the pupae of *Culex pipiens* L. (Rodríguez-Prieto et al. 2006). This behavior may have a cost in terms of fitness, as it increases mortality in larvae (Tuno et al. 2004) and reduces survival after eclosion (Lucas and Romoser 2001). However, for species living in very small habitats such as phytotelmata, diving behavior protects larvae and pupae from being washed away by raindrops (Romoser and Lucas 1999, Lucas and Romoser 2001).

Mosquitoes may also dive to avoid predators. However, backswimmers (*Notonecta* spp.) and the predacious mosquito *Toxorhynchites rutilus* (Coq.) are attracted to the movement of active mosquito larvae, and thus motion must be limited to avoid these predators (Sih 1979, Juliano and Gravel 2002). Although these results suggest that diving behavior is not effective against aquatic predators, it may function well against terrestrial or aerial predators that cannot enter the water. Minakawa et al. (2007) used a terrestrial predator, *Ochthera chalybescens* Loew, to test this hypothesis against *Anopheles gambiae* Giles. However, the study focused on third-instar mosquito alone, and the data provided was not sufficient for testing the hypothesis.

Because *An. gambiae* feeds at the water surface, it is a good organism with which to test the predator-avoidance hypothesis. Moreover, *An. gambiae* is a major malarial vector in Africa, which emphasizes the importance of understanding the ecology and evolution of this organism and its predators. Service (1971, 1977) identified several predators of *An. gambiae* in western Kenya using the serological method. Among these, wolf spiders (Lycosidae) were important predators of newly emerged the *An. gambiae* complex adults; however, the study did not examine whether these spiders prey upon larvae or pupae. Perevozkin et al. (2004) found that spiders belonging to the genus *Dolomedes* (Pisauridae) actively prey upon anopheline and culicine larvae in Russia. Wolf spiders (*Pardosa* spp.) did not seem to be active predators of mosquito larvae. However, that study was limited in that it only examined predatory ability against fourth instar. Diving ability also differs among the immature life stages of *An. gambiae* (Tuno et al. 2004), which may affect the predatory ability of wolf spiders.

We studied the predatory ability of the wolf spider *Pardosa messingerae* (Stand) on all immature life stages of *An. gambiae*. This spider is often observed on the water surface at breeding sites of *An. gambiae* in western Kenya. We compared diving responses among mosquito life stages and used these differences to test the predator-avoidance hypothesis.

## **Materials and Methods**

**Predatory ability.** We designed an observational study to determine whether *P. messingerae*

preys upon the larvae of *An. gambiae*. Adult and sub-adult spiders were collected from temporary pools in the Suba District of western Kenya which were known as breeding sites of the *An. gambiae* complex. *Anopheles gambiae* were obtained from the insectary at the International Centre of Insect Physiology and Ecology (ICIPE) at Mbita Point Research and Training Centre (Mbita, Kenya).

Ten spiders were selected at random and starved (water only) for 15 d prior to the experiment. Ten plastic containers (11.0 cm in diameter × 6.5 cm in height) were filled with distilled water to a depth of 1.0 cm. A wooden float (0.5 × 1.0 × 1.5 cm) was placed in each container as a scaffold for the spider. Ten fourth-instar *An. gambiae* and one starved spider were introduced into each container. We used fourth-instar mosquitoes because they are large enough for easy observation. Each container was covered with a piece of mosquito netting and maintained at 27°C for 24 h in a laboratory at ICIPE. During the experiment, we observed predatory activity for 1 h. At the end of the experiment, we counted the number of missing mosquito larvae in each cup.

We also tested whether wolf spiders are able to prey upon first-instar mosquitoes. Because first-instar mosquitoes are small, first-instar spiderlings were used in place of adults. We also performed a parallel experiment without predators as a control for cannibalism (Koenraadt and Takken 2003). The spiderlings emerged from egg sacs in the laboratory and were starved (water only) for 1 week prior to the experiment. Predatory ability on 20 first-instar *An. gambiae*

was assessed as previously described.

**Effects of water depth and life stage on predation.** We examined whether water depth or immature mosquito life stage affected predatory ability. The effect of depth was measured using plastic cups (7.0 cm in diameter  $\times$  7.5 cm in height) filled to a depth of 3.0 cm (deep water,  $n = 24$ ) or 0.5 cm (shallow water,  $n = 24$ ). The deep water gave larvae adequate space to dive and avoid the predator, whereas diving was restricted in the cups with shallow water. The cups were kept in a water bath maintained at a minimum temperature of 27°C overnight. On the following morning, 20 first-instar mosquitoes and a wooden float (1.0  $\times$  1.5 cm) were placed into each cup. Spiders starved for 3 d prior to the experiment were introduced into 12 cups with deep water and 12 cups with shallow water; as a control, the remaining 24 cups contained only mosquitoes. Each cup was covered with a piece of mosquito netting and maintained at a minimum temperature of 27°C in a water bath. Although the water bath could not control maximum temperature, the maximum room temperature was 29.5°C, and the mean room temperature was 26.6°C during the experimental period. After 24 h, the number of larvae missing from each cup was recorded. The same procedure was used for all other immature mosquito life stages, including pupae. In total, we had 20 treatments (two water depths  $\times$  presence or absence of predator  $\times$  five immature prey stages) with 12 replicates in each treatment. However, because of a shortage of spiders, only 11 replicates were performed for the second and fourth instar. In the treatments using pupae, some individuals emerged as adults

during the experimental period and were consumed by the spiders; for this reason, we counted total pupal skins instead of individual adult survivors.

**Diving responses in mosquito larvae.** Differences in diving responses were examined among the five immature life stages of *An. gambiae*. Diving responses was assessed based on how many mosquitoes dove and for how long. We estimated the number of diving individuals in still water and after a disturbance (a drop of water on the surface). Twenty larvae from each instar or pupae were placed in a plastic cup filled with distilled water to a depth of 3.0 cm. After allowing the cup to rest for 10 min, the number of diving mosquitoes was counted. We repeated this procedure six times with an interval of 10 min between trials. Three trials were performed for each condition (still versus disturbed), and the order of these trials was selected at random. Ten replicates or individual cups were performed for each life stage.

To assess diving duration, a larva or pupa was placed in a conical tube (2.5 cm in diameter × 9.5 cm in height) filled to a depth of 7.0 cm with distilled water. After allowing the tube to rest for 10 min, water was dropped directly on the mosquito from a height of 2.0 cm using a pipette. If the mosquito did not dive after the first drop, more drops were added until the mosquito dove (up to a maximum of five drops). The duration of diving was timed until the mosquito returned to water surface by itself. The procedure was repeated three times for each individual with a 10-min interval between trials, and the mean diving duration was calculated. This procedure was repeated with 20 individuals for each life stage.

**Data analyses.** A *t*-test was used to compare the number of missing first instar mosquitoes between treatments with or without spiderlings. Three-factorial analysis of variance (ANOVA) was used to examine effects of three factors (water depth, presence of predator, and mosquito life stage) and their combined effects on the number of missing mosquitoes.

Missing larvae in the cups without predators indicated that cannibalism occurred.

Cannibalism is common among *An. gambiae* larvae, and it is the only plausible explanation for missing mosquitoes in cups without predators (Koenraadt and Takken 2003, Minakawa et al. 2007). Because cannibalism may lead to the overestimation of predation intensity, we performed a separate analysis to examine effects of life stage and water depth, excluding the effect of cannibalism. We found that the intensity of cannibalism varied among life stages and water depth. We assumed that similar levels of cannibalism occurred in cups containing spiders, and we subtracted the total number of mosquitoes missing from cups without spiders from those with spiders for each treatment. We then calculated the proportion of missing mosquitoes at each depth for each life stage and compared the proportions among life stages using a chi-squared test. The level of significance was adjusted to 0.01 using Bonferroni method.

Split-plot ANOVA was used to detect differences in the number of diving individuals in still versus disturbed water for each life stage. Differences in the mean diving duration among life stages were also examined using Kruskal-Wallis test because of heteroscedasticity. The Tukey–Kramer test was used as a post-hoc multiple comparison test for ANOVA, and the



Tukey-type non-parametric multiple comparison test was used for Kruskal-Wallis test. When an interaction was statistically significant, we examined the interaction in more detail using the test of simple effects (Winer 1971).

## Results

**Predatory ability.** Seven of the ten containers had fewer than ten fourth-instar mosquitoes after 24 h in the presence of a wolf spider. The number of missing larvae ranged from zero to eight, and the mean was 2.7 (SE = 0.91) of ten larvae. We also noticed that head capsules and hairs from mosquito larvae had been discarded on the bottom of the containers. During the 1-h observation period, three spiders held mosquito larvae in their chelicerae and ingested them (Fig. 1).

In the experiment using first-instar spiderlings, two spiders died and were removed from the analysis. The mean number of missing larvae was 7.1 (SE = 0.64) of 20 larvae in containers with spiders and 0.1 (SE = 0.06) without spiders. This difference was significant ( $t = 7.934$ ,  $df = 36$ ,  $P < 0.001$ ).

**Effects of water depth and life stage on predation.** Three-factorial ANOVA revealed that the model was significant (Table 1). The main effects of predator and mosquito life stage were significant, whereas the effect of water depth was not. In addition, the interaction between predator and mosquito life stage was significant, suggesting that predation intensity differed

among prey life stages. The test of simple effects revealed that the difference in the number of missing mosquitoes between the cups with or without spiders was insignificant for first instar; in contrast, the cups with spiders showed significantly more missing mosquitoes in other larval instars and pupa (Fig. 2a). The interaction between depth and life stage was also significant, suggesting that the effect of depth differed among life stages. The test of simple effects revealed that the number of missing larvae in between shallow and deep water was significantly different in second and third instar (Fig. 2b). In contrast, the interaction between predator and depth was insignificant.

When the effect of cannibalism was excluded, the proportion of cannibalism corrected mortality differed significantly among life stages ( $\chi^2 = 12.40$ ,  $df = 4$ ,  $P = 0.015$ ), suggesting that the intensity of predation in deep versus shallow water differed according to prey life stage (Table 2). Furthermore, we examined the proportion of cannibalism corrected mortality in deep versus shallow water for each life stage using a chi-squared test. The analysis revealed that the corrected mortality was significantly lower in deep water than in shallow water for the second instar ( $P < 0.01$ ); no significant difference was observed for the remaining life stages after Bonferroni correction.

**Diving ability of mosquito larvae.** The split-plot ANOVA revealed that disturbance and larval life stage were important factors for the number of diving larvae ( $F_{1, 45} = 111.39$ ,  $P < 0.001$  for disturbance;  $F_{4, 45} = 39.17$ ,  $P < 0.001$  for life stage;  $F_{4, 45} = 6.65$ ,  $P < 0.001$  for interaction). The

mean number of diving larvae was 3.1 (SE = 0.31, range = 0 to 6.3; 15.5% of total larvae) in still water and 7.2 (SE = 0.31, range = 0 to 12.2; 36.0%) in disturbed water. The post-hoc test revealed that the number of diving larvae was significantly higher in disturbed water compared to still water for all larval stages, and second instar dove more frequently than any other instar (Fig. 3).

Kruskal-Wallis test revealed that diving duration differed significantly among life stages ( $\chi^2 = 63.27$ ,  $df = 4$ ,  $P < 0.001$ ). Furthermore, diving duration was inversely proportional to mosquito age (Fig. 4). The post-hoc test revealed that diving duration was significantly longer during the first and second instars compared to fourth instar and pupae.

## Discussion

The results of this and previous studies support our conclusion that a wolf spider, *P. messingerae*, preys on all life stages including adults of *An. gambiae* (Service 1971, 1977). Although predation intensity was low for first instar, separate experiments showed that the starved spiders captured nearly 50% of second, third, and fourth instar within 24 h. Although adult and sub-adult spiders both had difficulty capturing first instar regardless of water depth, first-instar spiderlings were able to capture this instar much more effectively. Our results cannot be directly extrapolated to describe the predatory capacity of this spider in nature, but they suggest that wolf spiders are at least partly responsible for the high mortality (over 90%)

observed in immature the *An. gambiae* complex (Service 1971, 1977).

Furthermore, our study shows the spider had difficulty capturing pupae regardless of water depth. Pupae are less active, which may draw less attention from this predator. A spider is considered to be especially attracted to a moving prey. In addition, pupal ability to move quickly and their round shape may also make pupae more difficult to capture. This is consistent with other studies showing that *O. chalybescens*, another important predator, has difficulty capturing the pupae of *An. gambiae* (Minakawa et al. 2007).

The number of missing mosquitoes increased from the first to fourth instar in cups containing deep water and the predator; at the same time, the duration of diving decreased, and the number of diving individuals decreased from the second to fourth instar. However, we did not observe a relationship between the number of missing mosquitoes and diving performance in cups containing shallow water where diving was restricted. If predatory ability depended on the body size of prey alone and not diving behavior, the number of larvae missing from the shallow water should have increased in a similar manner. After excluding the effects of cannibalism, we observed that the corrected mortality was significantly greater in shallow water compared to deep water for the second instar. These results suggest that differences in diving ability among instars affect the success of predation in wolf spiders. Thus, mosquito larvae avoid the spider by diving more frequently and for longer periods; predator avoidance was most successful in second instar. This study is the first to show that diving behavior allows

mosquitoes to escape from a terrestrial predator, and it provides evidence to support the predator-avoidance hypothesis.

Although the mortality was significantly greater in shallow water compared to deep water for the second instar, the three-way ANOVA revealed insignificance of water depth. Poor diving performance of older instar individuals and pupae might reduce the effect of depth in the statistical analysis. Another possible reason is that the water depth was not enough for older mosquitoes to escape from the spiders. In nature, many breeding sites are deeper than 3 cm, and the importance of diving for avoiding predators may become more pronounced. While avoiding predators by diving deeply, the behavior may increase another cost for survival (Tuno et al. 2004).

The observed decrease in diving duration according to age may be the result of a declining capacity for cutaneous respiration in older individuals (Thomas 1950, Kasap 1981). In young larvae, the surface-to-volume ratio is high, and a greater portion of the larval oxygen requirements can be met by surface diffusion through the cuticle. As size increases, the surface-to-volume ratio decreases and the larva requires more oxygen to meet the increasing demand. For this reason, older larvae must spend more time at the surface to draw oxygen through a respiratory siphon. Thomas (1950) showed that although first and second instar can survive when held underwater by a layer of fine netting, third and fourth instar cannot. In pupae, the presence of a ventral air space prevents them from diving for a long period, unless

buoyancy is lost through compression by the surrounding water. In addition, the development of the thick pupal cuticle decreases the efficiency of cutaneous respiration (Clements 1999), which may also explain why diving duration decreases with age. However, fewer first instar dove in comparison to second instar, which may be because many first instar are too small or weak to break the surface tension of the water (Clements 1999).

A previous study found that diving is not an effective means for third instar mosquitoes against the shore fly *O. chalybescens* (Minakawa et al. 2007). This result is consistent with our own, in that diving frequency and duration decrease with age. In contrast, the number of missing larvae gradually decreased from first instar to pupae in the shore fly study. One plausible explanation for this discrepancy is the difference in feeding habits between shore flies and wolf spiders. The shore fly captures prey using unique sickle-shaped front legs that seem to function to grab moving prey such as very active early instar mosquitoes. In contrast, the predatory capacity of the spider seems to depend upon the size and evasive ability of the prey.

In contrast to *An. gambiae*, *C. pipiens* larvae cease diving in the presence of backswimmers (Sih 1979, 1986). Similarly, *Aedes triseriatus* (Say) larvae tend to remain near the water surface for longer periods after they spend three generations with their predator, *T. rutilus* (Juliano and Gravel 2002). These results indicate that ceasing diving is an effective way to escape from aquatic predators for these mosquitoes. We suspect that the difference shown in predator-avoidance behavior between anopheline and other species is because of differences in

feeding place (in water or on water surface) of the mosquitoes and predators. *Anopheles gambiae* is a surface feeder and is thus much more vulnerable to terrestrial and aerial predators; diving behavior may have evolved in response to this pressure. In contrast, the larvae of *C. pipiens* and *Ae. triseriatus* forage on the bottom and move to the surface for respiration, which makes them more vulnerable to aquatic predators than terrestrial predators; thus, ceasing movement is a more effective way for these mosquitoes to avoid aquatic predators.

Interestingly, studies of *C. pipiens* found that pupae dive and remain below when a shadow repeatedly crosses the surface (Rodríguez-Prieto et al. 2006), suggesting that the diving behavior of *C. pipiens* plays a role in escaping from aerial or terrestrial predators. This study implies that the larvae and pupae of anophelines may become quiescent near the water surface when an aquatic predator is present. Thus, immature mosquito life stages are under at least two opposing selective pressures, which may drive each species to evolve a particular facultative behavior (Juliano and Gravel 2002).

Our study indicates some important implications for controlling malaria vectors. As the wolf spider is an important predator of the *An. gambiae* complex, application of insecticides to the spider's habitat should be avoided. This spider may become a good candidate of biological control agent. Introducing multiple predator species with different predatory strategies will be more effective for biologically controlling immature mosquitoes than a single species, because the predator-avoidance performance varies among the developmental stages of immature

mosquitoes, and mosquitoes may alter predator-avoidance strategies according to predator types (terrestrial or aquatic).



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**Table 1. Three-factorial ANOVA for the effects of immature life stage, predator presence, and water depth on the number of missing *An. gambiae*.**

Factor	df	SS	<i>F</i>	<i>P</i>
Immature stage	4	1043.11	26.53	<0.001
Predator	1	2240.99	227.99	<0.001
Depth	1	4.41	0.45	0.504
Immature stage × predator	4	437.82	11.14	<0.001
Immature stage × depth	4	117.93	3.00	0.020
Predator × depth	1	0.97	0.10	0.750
Immature stage × predator × depth	4	42.95	1.09	0.360

**Table 2. Cannibalism corrected mortality for each immature life stage in deep water versus shallow water.**

Stage	Depth	Total mosquito	Cannibalism	Corrected total mosquito	Corrected mortality (%) <sup>a</sup>	$\chi^2$
First	Deep	240	7	233	21 (9.0)	0.12
	Shallow	240	6	234	19 (8.1)	
Second	Deep	220	23	197	59 (29.9)	10.04*
	Shallow	220	36	184	84 (45.6)	
Third	Deep	240	26	214	100 (46.3)	1.20
	Shallow	240	2	238	99 (41.6)	
Fourth	Deep	220	16	204	120 (58.8)	3.84
	Shallow	220	34	186	91 (48.9)	
Pupa	Deep	240	0	240	51 (21.3)	5.75
	Shallow	240	0	240	74 (30.8)	

<sup>a</sup> To exclude the effect of cannibalism, the total number of missing mosquitoes from the cups without spiders was subtracted from the total number of missing mosquitoes in the cups with spiders. After excluding the effect of cannibalism, chi-squared tests were used to detect differences in the number of missing mosquitoes.

\* Statistically significant after Bonferroni correction,  $P < 0.01$ .

**Figure 1.** A wolf spider *Pardosa messingerae* feeding on an *Anopheles gambiae* larva. The arrow indicates the head of the captured mosquito larva.

**Figure 2.** (a) Mean number  $\pm$  standard error of missing mosquitoes in cups with (black circles) or without (black squares) starved spiders joined in shallow and deep water. (b) Mean number  $\pm$  standard error of missing mosquitoes in deep (black circles) or shallow water (black squares) joined with and without starved spiders. Asterisks indicate significant differences between the two treatments for each mosquito life stage.

**Figure 3.** Mean number  $\pm$  standard error of diving immature mosquitoes in still (black circles) versus disturbed water (black squares). Asterisks indicate significant differences between the two treatments at each stage. Identical letters indicate non-significant differences among stages.

**Figure 4.** Mean diving duration  $\pm$  standard error for immature mosquitoes. Identical letters indicate non-significant differences among stages.





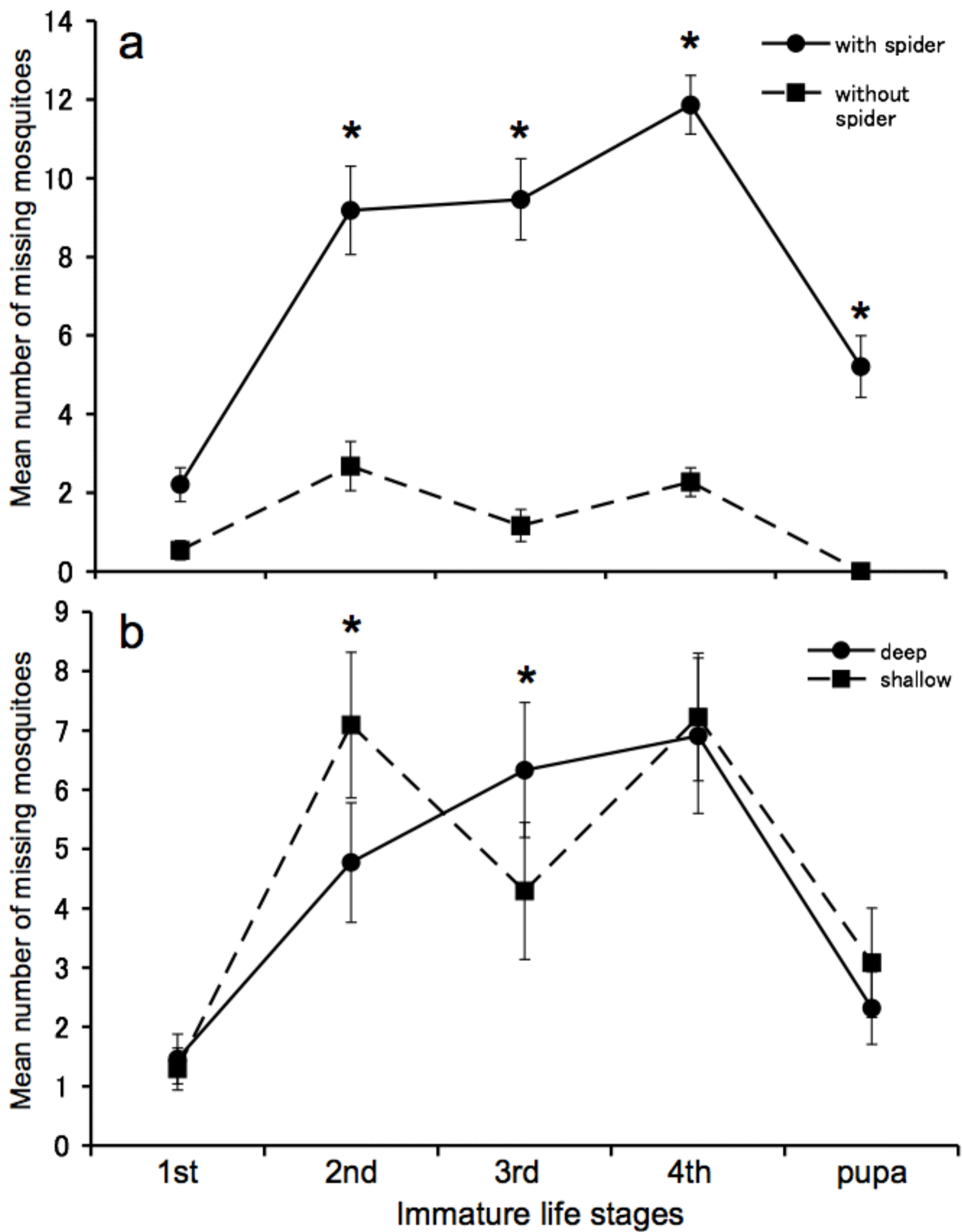


Fig. 2

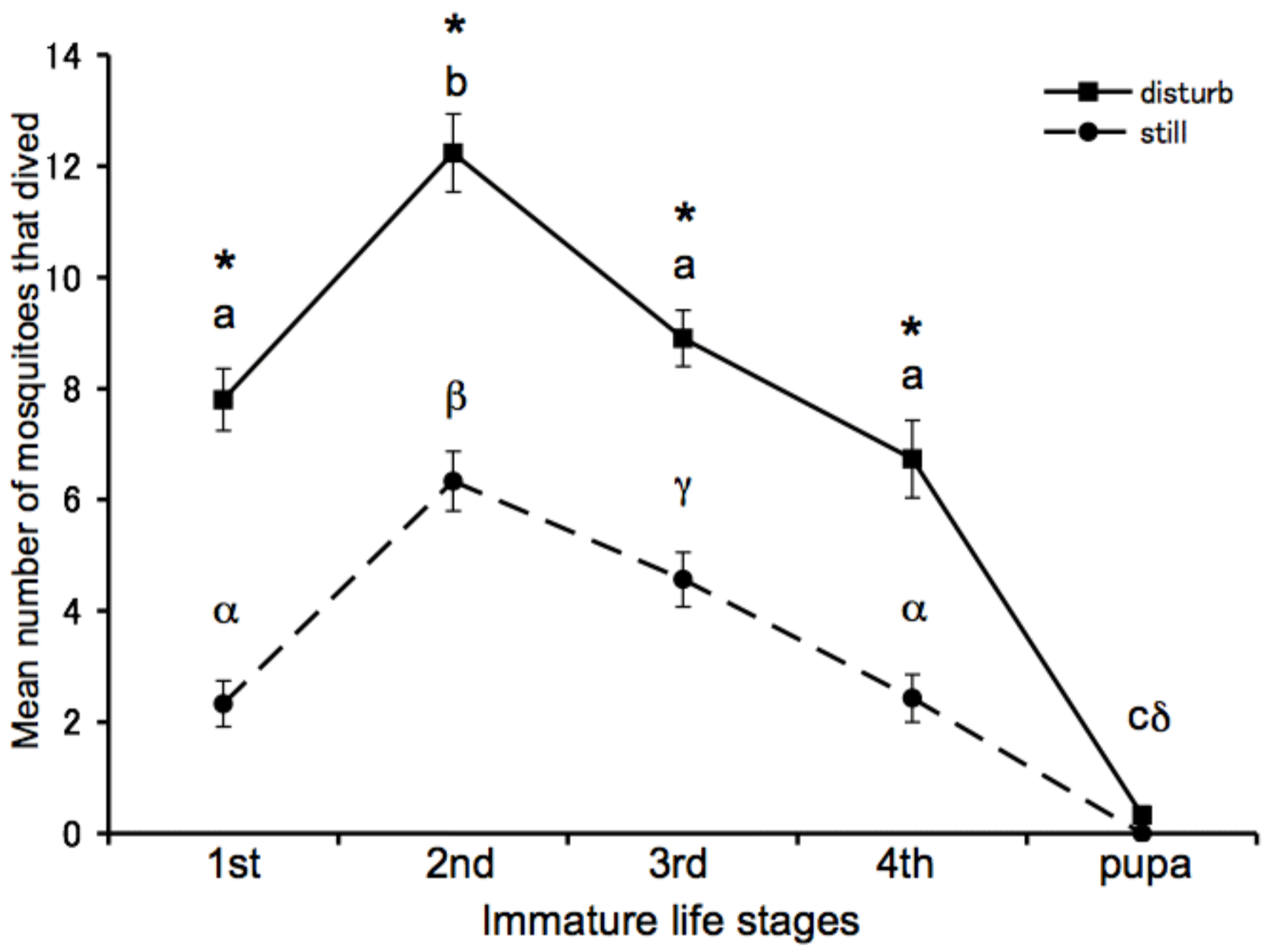


Fig. 3

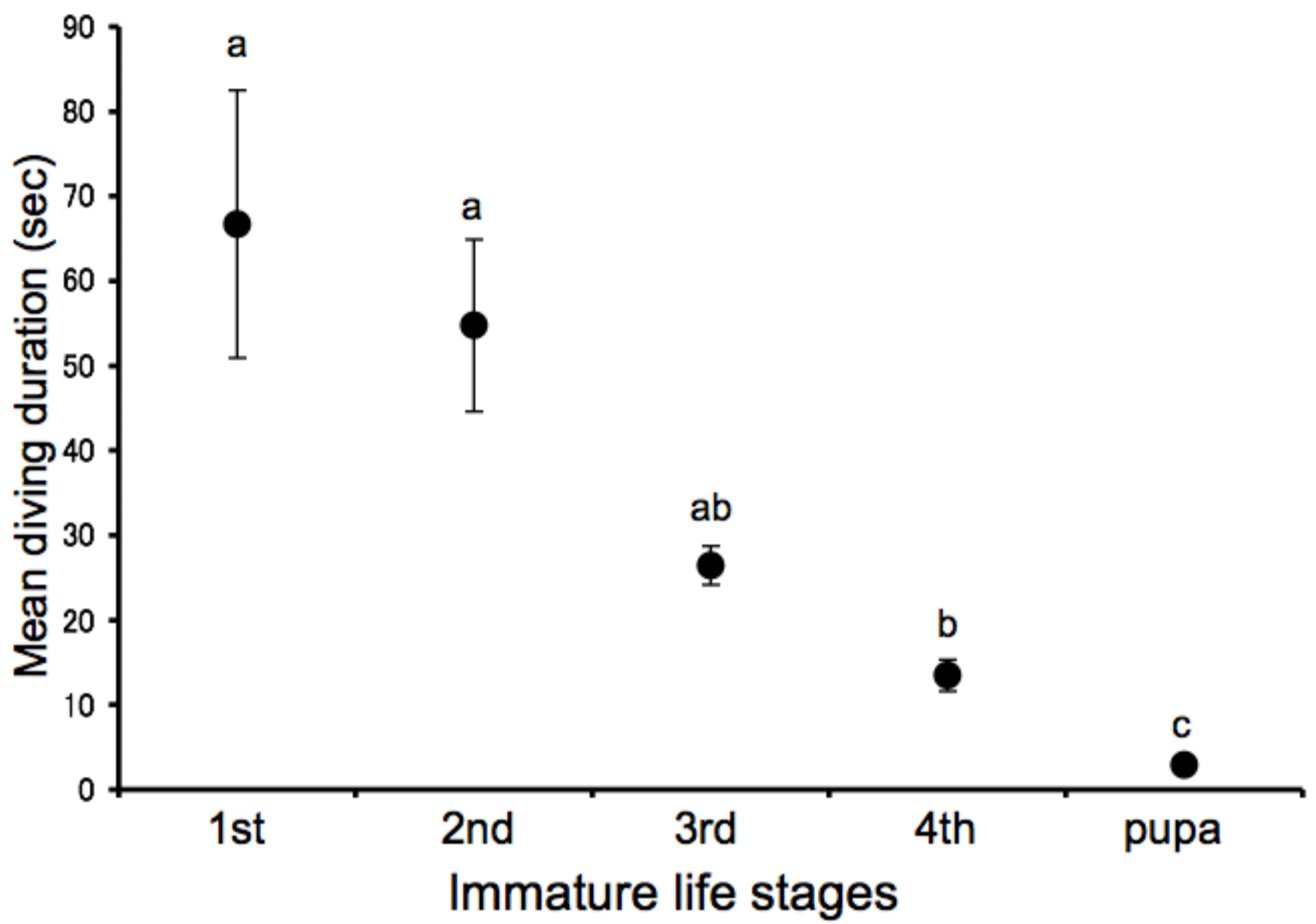


Fig.4