



Mating behavior and effects of light on the fairy shrimp *Branchinella kugenumaensis* (Ishikawa, 1895): a vision dominated mating system

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ABSTRACT: Large branchiopods face time constraints in reproduction efficiency due to their living in temporary environments. Efficiency on mate locating is especially important for sustaining their populations. Light has been demonstrated as an important factor influencing mating success for anostracans, but detailed information is lacking. *Branchinella kugenumaensis* tends to aggregate at water surface. We hypothesized that this tendency is related to necessity of light for mating. We observed *Branchinella kugenumaensis* mating behavior, which contained 6 elements: orientation, station taking, amplexus, intromission, copulation and disengagement. In addition, we compared the first 3 elements of behavioral responses of focal males toward three types of potential mates (receptive females, non-receptive females and other males) in both light and dark conditions. We found that light effects were significant in all 3 behavioral elements, regardless of potential mate types. Therefore, we suggest that male *B. kugenumaensis* relies on vision to assess potential mates.

KEY WORDS: Anostraca, *Branchinella kugenumaensis*, Mate locating, Taiwan, Temporary wetlands, Visual systems.

INTRODUCTION

When reproduction time or opportunities are limited, searching for mates is more beneficial to reproduction than dominating resources (Scott, 1974). In most dioecious systems, males tend to give low reproductive investment for each mating but take high reproductive rate (Gwynne, 1991; Clutton-Brock and Parker, 1992, 1978), thus mate searching behaviour is usually performed by males. Conversely, due to greater parental investment (Gwynne, 1991), the female should produce some cues that reveal their location and identify them as suitable mates when they are receptive. Mate searching behavior is thought to be under strong selective pressure since successful sexual reproduction requires accurate mate locating (Parker, 1978). Mechanisms of searching behavior and mate discrimination are elaborate in crustaceans, with sexual communication cues manifest as visual (Goulden, 1968), chemical (Larsson and Dodson, 1993; Snell and Morris, 1993; Van Damme and Dumont, 2006), or both (Hughes, 1996; Díaz and Thiel, 2004; Saunders *et al.*, 2010). Hence, identifying communicational cues during mating is important for understanding mating systems and environmental adaptations.

Large branchiopods usually inhabit temporary wetlands characterized by stochastic drought and inundation (Williams, 1996; Schwartz and Jenkins, 2000). Living in such environmental instability and unpredictability, animals usually perform short life cycle and early reproduction during the short time

frame of hydroperiod to avoid abortive hatchings, which may reduce the population (Brendonck, 1996; Brendonck *et al.*, 2000; Ripley *et al.*, 2004; Huang *et al.*, 2011). Therefore, large branchiopods demonstrate early hatching, rapid growth, and frequent reproduction during each hydroperiod (Wiggins *et al.*, 1980; Brendonck *et al.*, 2000; Huang *et al.*, 2010). Population densities of mature large branchiopods are generally high when the hydroperiod terminates. In this situation, resource and mate defense is energetically unfeasible and male competition intensity increases (Emlen and Oring, 1977). It can be expected that efficient mating systems should play important role for crustaceans living in unstable environment. Previous large branchiopod studies mainly focused on life histories (Hildrew, 1985; Zucker *et al.*, 2001; Huang *et al.*, 2010; Vanschoenwinkel *et al.*, 2010), while mating behavior studies were mostly limited to clam shrimps (i.e., *Eulimnadia texana* (Packard, 1871) (Knoll, 1995; Knoll and Zucker, 1995; Medland *et al.*, 2000; Weeks and Benvenuto, 2008; Benvenuto and Weeks, 2012), *Cyzius grubei* (Simon, 1886) (Pérez-Bote, 2010), and *Lynceus brachyurus* Muller, 1776 (Sigvardt and Olesen, 2014)). The few studies on anostracan mating behavior described and divided the system into 6 elements: detection and orientation, station taking, amplexus, intromission, copulation, and disengagement (Wiman, 1981; Belk, 1991; Rogers, 2002). Males must detect female cues for successful location. In *Eubranchipus serratus* Forbes, 1876, it was demonstrated that visual cues alone were sufficient in leading males to exhibit



mate selection behavior (Belk, 1991). Females were not fertilized in dark environments. Nevertheless, the exact phase does vision affect is not clear yet.

Branchinella kugenumaensis (Ishikawa, 1895) is widely distributed in eastern Asia (Brendonck and Belk, 1997; Yamada, 1999; Grygier *et al.*, 2002; Huang *et al.*, 2010; Rogers *et al.*, 2013). Previous studies on *B. kugenumaensis* were mainly focused on life history (Huang *et al.*, 2010, 2011), spatial distribution (Wang *et al.*, 2012; Wang *et al.*, 2014), and hatching phenology (Wang and Chou, 2015). In Taiwan, *B. kugenumaensis* inhabits Siangtian Pond, a temporary pool located in northern Taiwan. Siangtian Pond's hydroperiod usually lasts 2-3 weeks and the basin can reach 6 m in depth (Huang *et al.*, 2010), thus vertical light penetration can be limited. *Branchinella kugenumaensis* requires nine days to become sexually mature (Huang *et al.*, 2010) and tends to aggregate at the pool surface (Wang *et al.*, 2012). This behavior may be related to a necessity of good light for mate recognition. Therefore, we observed *B. kugenumaensis* mating behavior and examined potential light effect on their mating performance.

MATERIALS AND METHODS

Study animals and culture conditions

Branchinella kugenumaensis dormant eggs were collected in sediment during the dry phase from Siangtian Pond, Yangmingshan National Park, Taiwan (N:25°10'26", E:121°29'56"; Huang *et al.*, 2010). Eggs were hatched in opaque plastic buckets (22 cm diameter, 28 cm high) under 22 ± 2 °C water temperature with sediment from their natural habitat and a yellow fluorescent lamp for lighting. (Yellow light promotes algal growth, providing more food for the shrimp). Juveniles were transferred to transparent glass tanks (34 x 22 cm, 25.5 cm height) with water temperature of 22 ± 2 °C and water volume maintained at eighty percent of the tank capacity until the shrimp were sexually mature. No food was added in culture.

Animals with body length greater than 1 cm were examined to determine maturity. Mature shrimp was defined as males with obvious frontal appendages and white semen visible in the vas deferens, and females with eggs in the brood pouch. The female reproductive cycle can be divided into 3 stages (Plodsomboon *et al.*, 2012); Stage 1: lateral pouches and ovisac empty; Stage 2: lateral pouches were full of unfertilized oocytes and ovisac empty; Stage 3: lateral pouches empty and ovisac full of eggs. Only females in Stage 2 were receptive to males (Belk, 1991; Rogers, 2002; Plodsomboon *et al.*, 2012) and were separated from males for 24 hours to ensure they would be receptive. Non-receptive females were in Stage 3.

Mating behavior observations

A courting male and a potential mate were moved from above glass tanks and placed in a cylindrical plastic container (6.5 cm radius), containing 350 ml (3 cm height), 22 °C (range from 20 – 29 °C) culture water. To prevent visual distractions, black cardboard was embedded against the inner surfaces of the container. The light source was a 14w, 60Hz white fluorescent lamp with a luminance of 281 lux. A camera (Nikon Coolpix P310) was mounted on a tripod 51 cm from the container to record mating activities. Only the first courtship event in each pair was recorded. Mating behavior stage duration was calculated by cutting the video into screenshots of 5 frames per second using The KMPlayer (v. 4.0; Pandora TV, 2013).

Illumination experiment

Study animals were transferred from above bigger glass tank into a glass tank (11 x 11 x 11.5 cm), with 650 ml (6 cm height), 22 °C (range from 20 – 29 °C) culture water. To prevent visual distractions, black cardboard was embedded against the inner surfaces. Two illumination conditions were used: light group with 1484 lux luminance provided by a 14w, 60Hz white fluorescent lamp; and dark group was controlled < 10 lux blue light provided by the same fluorescent lamp wrapped in blue cellophane. Blue cellophane was chosen because observation under blue light was easier than under red. A pretest was conducted, wherein no significant difference in frequencies of orientation, station taking, and amplexus between blue and red colors were observed (Mann-Whitney U test, $p > 0.05$ for all behaviors; Appendix 1).

We observed focal male mating behavior when presented with three types of potential mates: receptive females (RF), non-receptive females (NF), and males (M). Study animals were placed in the tank for least 2 minutes for environmental habituation prior to the observation period, and each observation period lasted for 10 minutes. One pair was examined per observation. Because when the mating courtship progress to the third stage, amplexus, which is quite brief (around 1-3 seconds only), and always followed by later three behavioral stages/elements. Therefore, we used performance of the first 3 elements to test the illumination effect. Frequency of the first three mating behavioral elements (orientation, station taking, and amplexus) by the focal males were counted. The definitions are presented in Table 1, following Wiman (1981), Belk (1991), and Rogers (2002). To prevent confusion, the cercopods of male potential mates were clipped. Males with clipped cercopods did not show any abnormal behavior during the pretest observations. All amplexus movement was counted regardless if males succeeded or not. If female receptivity changed during observations (i.e., non-receptive females released their

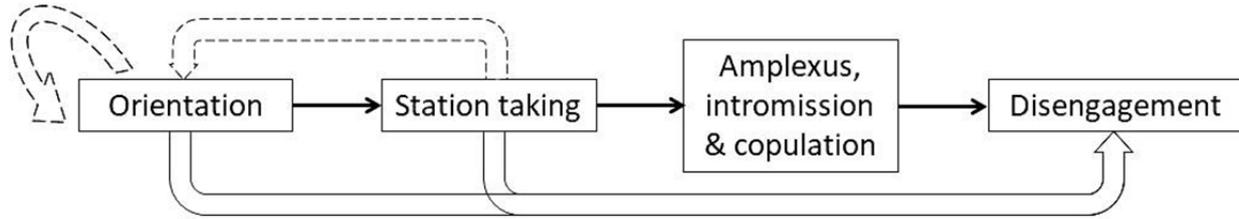


Fig. 1 *Branchinella kugenumaensis* mating behavior process. Black arrows represent the general pattern. Solid and dotted hollow arrows represent two non-general situations caused by mate fleeing.

eggs, or copulation succeeded and receptive females became fertilized), the observations were terminated and the data excluded. We tested 90 pairs of 30 replications for each of 3 potential mate encounter types. Each pair was conducted in both light and under dark conditions, and the treatment sequence was random. Each anostracan individual was not reused between pairs.

Table 1. Definitions of orientation, station taking, and amplexus, based on past behavioral description (Wiman, 1981; Belk, 1991; Rogers, 2002)

Behavior	Definition
Orientation	Male orients to a potential mate
Station taking	Male keeps head around potential mate genital segments and sustains this position more than 1 second. If male drifts behind potential mate cercopods, it is considered that station taking terminated
Amplexus	Male extends frontal appendage and amplexes potential mate with second antennae

Statistical analysis

IBM SPSS Statistics (v. 20.0; IBM Corporation, 2011) software was used for the statistical analyses. Behavioral frequency means were compared by Wilcoxon matched paired signed rank test because of abnormality (Kolmogorov-Smirnov test, $p < 0.001$ for all the behavior under both light and dark conditions).

RESULTS

Mating behavior

Branchinella kugenumaensis mate identification behavior was initiated by the male. Mating behavior is divided into 6 stages of orientation, station taking, amplexus, intromission, copulation, and disengagement (Rogers, 2002). We recorded 135 station taking events, wherein amplexus was attempted 32 times, and successful intromission occurred 6 times. Males attempted amplexus with 42.2 % of receptive females, 13.3 % of non-receptive females, and 15.6 % of males. Intromission occurred only with receptive females.

In the orientation stage, males oriented themselves to potential mates so that they could inspect the potential mate genital segments. This behavior usually accompanied an accelerating swimming speed caused by

telson movement. In the next stage of station taking, males maintained their heads above potential mate genital segments. The position was maintained with the male following every movement of the potential mate; if the pairs were stationary, the male's cercopods were usually spread about 160° , which assisted the males to hold position. Station taking duration varied greatly, due to non-stop potential mate activity (Table 2). Period duration of station taking was defined from when a courting male's head first reaches the potential mate's genital segments, to when amplexus occurred or when the courting male turned away from the potential mate.

After station taking, males moved below the female, extended their frontal appendage and amplexed the potential mates. Amplexus was always followed by rapid attempted intromission, wherein the male arced its body in an attempt to insert his gonopod into the female gonopore. In most cases, females would struggle and then males were unable to intromit successfully. If successful intromission occurred, then sperm was transferred to female, and the pair sank to the bottom for several seconds (Table 2). Ultimately, the two shrimp disengaged and returned to normal swimming movement, or the male attempted another mating event.

Figure 1 presents the pathway of the mating behavior system. Black arrows represent the general pattern which including orientation, station taking, amplexus (followed immediately by intromission and copulation), and disengagement. Sometimes potential mate rejects the male after orientation or during station taking. In this situation, the male may enter the next orientation phase by approaching the potential mate again (dotted hollow arrows). Conversely, if the male rejects the potential mate, the process was treated as the disengagement stage (solid hollow arrows).

Table 2. Time duration (seconds) of *Branchinella kugenumaensis* station taking and successful intromission behaviors.

	Mean \pm SD	Max	Min	N
Station taking	5.6 \pm 5.0	33.6	1	135
Intromission	2.1 \pm 0.7	3.4	1.6	6

Illumination effects

Mean (\pm SD) frequencies of three mating behavior types (orientation, station taking, and amplexus) under light and dark conditions are presented in Table 3.



Mating behavior frequency was significantly lower in dark than that in light condition regardless of behavior type by an order of magnitude (Fig. 2). Specific data on comparisons in male behavioral responses toward the 3 types of potential mates (receptive females, receptive females, and males) under two illumination conditions are listed in Table 4. No matter what kind of potential mates were provided, focal males responded in similar trend. All mating behavior frequencies under dark conditions were significantly lower than those under light conditions (Wilcoxon matched paired signed rank test, $p < 0.01$ for each comparison).

Table 3. Frequency (mean \pm SD) of *Branchinella kugenumaensis* orientation, station taking, and amplexus under light and dark conditions. All behaviors were significantly lower in dark condition (Wilcoxon matched paired signed rank test). Sample size was 90 trials for each light condition

	Light	Dark	Z
Orientation	9.0 \pm 8.2	0.8 \pm 1.7	7.2**
Station taking	5.1 \pm 6.1	0.3 \pm 0.8	6.7**
Amplexus	1.3 \pm 2.2	0.1 \pm 0.2	5.8**

** represents p value < 0.001

Table 4. Frequency (mean \pm SD) of *Branchinella kugenumaensis* orientation, station taking, and amplexus performed by males toward receptive females (RF), non-receptive females (NF), and males (M) under light and dark conditions. Comparisons used Wilcoxon matched paired signed rank test. The sample size was 30 trial for each potential mate type.

		RF		NF		M	
		Mean	Z	Mean	Z	Mean	Z
Orientation	Light	11.3 \pm 9.1		7.4 \pm 8.1		8.2 \pm 7.1	
	Dark	0.7 \pm 1.4	4.5**	0.3 \pm 0.8	4.0**	1.5 \pm 2.4	4.0**
Station taking	Light	8.1 \pm 7.8		3.7 \pm 4.9		3.4 \pm 3.9	
	Dark	0.4 \pm 1.0	4.5**	0.1 \pm 0.4	3.5*	0.4 \pm 0.8	3.7**
Amplexus	Light	2.6 \pm 3.0		0.7 \pm 1.5		0.7 \pm 1.0	
	Dark	0.1 \pm 0.3	4.1**	0.0 \pm 0.2	2.7*	0.0 \pm 0.0	3.1*

* represents p value < 0.01; ** represents p value < 0.001

DISSCUSSION

In *Branchinella kugenumaensis* mating system behaviors, when males detected potential mates, they oriented to the mate, following behind and station taking for a period time. If regarding the potential mate as suitable, males extended frontal appendages and attempted to amplex the mate with second antennae. An intromission attempt always accompanied amplexus, and if successful, sperm was transferred. The two fairy shrimps then disengaged and returned to normal swimming. In order to overcome the difficulty of observing *B. kugenumaensis* behavior under dark conditions, we used cellophane to control the light intensity so that “dark” conditions were bright enough for observer’s eyes but not necessarily for the anostracan compound eyes. In this way, we explored their dark environment behaviors. Intromission and

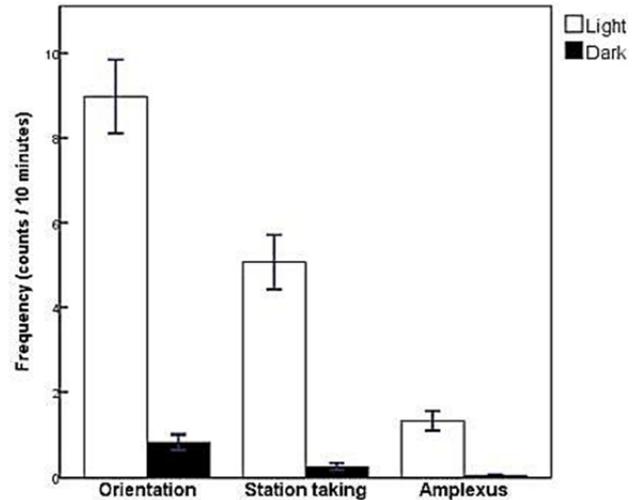


Fig. 2 Frequencies of *Branchinella kugenumaensis* orientation, station taking, and amplexus under light and dark conditions. All behaviors were significantly lower in dark condition (Wilcoxon matched paired signed rank test, $p < 0.001$ for each comparison). Sample size was 90 trials for each light condition. Error bars represent standard error of means.

copulation behavior were not measured in these experiments because they were so very rapid and hard to observe. In the dark environment, male *B. kugenumaensis* continued swimming, trying to search for potential mates, rather than staying stationary, however, they seldom exhibited any mating behaviors to potential mates, even if the mate was a suitable, receptive female.

The mating behavior of *B. kugenumaensis* is similar to descriptions for *Streptocephalus* and *Thamnocephalus* (Wiman, 1981; Rogers, 2002). When males detected a potential mate, orientation was accompanied by telson movement, apparently, to aid males in getting close to mates rapidly. Cues for mate locating might be visual or vibrational (Wiman, 1981; Belk, 1991). In this study, orientation was seldom exhibited in dark environments, indicating that visual cues should play key role in mate detection and locating, which is in accordance with the result of Belk (1991). In branchiopods, visual cues are also hypothesized for mate locating in some cladocerans (Goulden, 1968). Visual cue utilization in aquatic crustaceans is restricted to species inhabiting clear (Christy and Salmon, 1991; Hughes, 1996) and shallow (Barlow *et al.*, 1982) environments. Since vision is expected to work only in conditions with sufficient light and better visibility (Díaz and Thiel, 2004).

When environments are turbid, usually true for many anostracan habitats, visual may be aided or replaced by other cues (Belk, 1991). Chemical signals were described to involve in sexual communication for many aquatic crustaceans, such as rock shrimps (Díaz and Thiel, 2004), snapping shrimps (Hughes, 1996), horseshoe crabs (Saunders *et al.*, 2010), copepods



(Snell and Morris, 1993), and cladocerans (Van Damme and Dumont, 2006). In large branchiopods, clam shrimps *C. grubei* and *E. texana*, visual cues were hypothesized to be replaced by chemical cues (Medland *et al.*, 2000; Pérez-Bote, 2010). Anostracan chemical cues were demonstrated in *Artemia* mating (Tapia *et al.*, 2015), wherein virgin males were placed in water from cultures with females. Focal males exhibited the same frequency of searching behaviors as if females had actually been present, as opposed to significantly lower frequencies in the control male population. Their result showed that searching behavior was affected by the chemical cues released by females. However, chemical cue utilization has never been tested on *B. kugenumaensis*. Nevertheless, we do not expect chemical cues for locating mates will be significant, although we cannot exclude its existence. Since they are inhabitants of temporary environments, they usually mature at the end of the hydroperiod. As the water shrinks, the available area to the shrimp is reduced, causing extremely high population densities and crowding. In this situation, chemical cues may not be necessary. Visual cues should be much more useful in this crowded environment.

Branchinella kugenumaensis (fairy shrimp) prefers the surface area and the edge regions in Siangtian Pond, whereas other two sympatric large branchiopods (clam shrimps: *Lynceus bififormis* and *Eulimnadia braueriana*) did not show this preference (Wang *et al.*, 2012). *Lynceus bififormis* was distributed over a wide range of depths with increasing dominance towards the deeper layers, while *E. braueriana* showed no significant pattern of vertical distribution. Since Siangtian Pond can fill to 6 m, light may not penetrate to the pool bottom sufficiently. *Branchinella kugenumaensis* has to stay in the brighter environment near surface to locate potential mates. Unlike other branchiopod crustaceans, anostracans have large and complicated compound eyes on stalks, which suggests the importance of vision in detecting their surroundings and mates. Conversely, for the co-occurring clam shrimps *E. braueriana* and *L. bififormis* in Siangtian Pond, vision is restricted by their carapaces, thus sensory cues may be replaced by chemical cues (Weeks and Benvenuto, 2008; Pérez-Bote, 2010), which would be more useful in the darker portions of the pool.

Mate discrimination is another component of successful mating. In crustaceans, the female receptive period is a transient phase (Jormalainen, 1998; Plodsomboon *et al.*, 2012). Mating with an unsuitable mate results in energy and time costs for species with short life spans. Thus, after locating a potential mate, a male needs to be able to discriminate suitability for mating success. Station taking appears to be the primary period for male discrimination of potential mates. In *B. kugenumaensis*, this phase continued for

several seconds, usually ending within 10 seconds. However, in some cases it was prolonged, which was usually due to the potential mate continuously swimming. It appeared that in a very short distance, males received discrimination cues. In Wiman's (1981) hypothesis, locomotory behavior, visual brood pouch cues, and pheromonal chemical cues may announce female receptivity. In *E. serratus*, behavioral difference exists between receptive and non-receptive females, and may make mating more efficient (Belk, 1991). Receptive females preferred swimming in the water column, where the males were, whereas non-receptive females usually stayed near the bottom. Staying in higher layers is hypothesized to increase the risk of exposure to avian predators. Therefore, for non-receptive females, the cost of staying in water column would be higher than fending males off. Chemical cues also proved to play an important role in mate discrimination (Tapia *et al.*, 2015), where *Artemia* males attempted to amplex both females and pseudo females at the same frequency, significantly higher than amplexing other males.

Amplexus duration varies depending on genus (Belk, 1991). In *B. kugenumaensis*, amplexus and intromission occurred almost simultaneously, and males always released mates immediately after intromission and copulation. In contrast, *Artemia* amplexus is prolonged (Belk, 1991; Tapia *et al.*, 2015) which is believed to represent mate guarding (Knoll, 1995; Weeks and Benvenuto, 2008). In the anostracan mating system of scramble-competition polygyny, reproductive success is mainly based on the number of inseminated females. If mating time is limited owing to transient hydroperiods and further restricted to daytime, males have two tactics to increase reproductive success: search for receptive females as much as possible or guarding one female (Weeks and Benvenuto, 2008). Mate guarding only exists when the benefit of guarding a female is greater than searching for other receptive females (Parker, 1974). When females were rare or difficult to find, male-male competition is expected to increase (Alonso-Pimentel and Papaj, 1996). Prolonging amplexus causes higher mating success than searching for another female. For *B. kugenumaensis*, enhancing searching ability in stochastic environments can insure high mating success since population density is high and females are abundant (Wickler and Seibt, 1981). The function of frontal and antennal appendages is part of the mate recognition system (Belk, 1984; Rogers, 2002). In *E. serratus*, males with altered antennal appendages had significantly lower rates of successfully mating with females than intact males did, but altering the antennal appendages did not affect the amplexus function.

Belk (1991) demonstrated the potential utilization of visual cues in anostracan mate recognition systems.



Our study is the first to test the detailed stages of a series of mating behavior affected by light. We found that under dark conditions, *B. kugenumaensis* males continued swimming and mate searching, rather than remaining stationary. Several mating events occurred, but only when males were very close to potential mates. In one instance amplexus was successful and the receptive female inseminated. Ability to mate in the dim environment was greatly circumscribed.

We cannot exclude the effect of chemical cues by this experimental design. Future work should focus on the effect of other sensory cues. The discovery of copulation in the dark may hint that multiple cues are utilized identify mates.

To conclude, *Branchinella kugenumaensis* mating behavior was observed, and was similar to the described general anostracan mating behavior pattern, detection and orientation, station taking, amplexus, intromission, copulation, and disengagement. Although males would try to search potential mates under dark conditions, they seldom exhibited mating behavior. We have demonstrated that visual utilization is a necessary condition for *B. kugenumaensis* to detect potential mates and may be associated with its surface aggregating preference.

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Appendix 1. Frequency (mean \pm SD) of *Branchinella kugenumaensis* orientation, station taking, and amplexus under red and blue light. No significant difference was found in any behavioral frequency between different color backgrounds (compared by Mann-Whitney U test, $p > 0.05$ for all behaviors). Sample size of red color was 6, and 23 for blue

	Red (N = 6)	Blue (N = 23)	U
Orientation	3.3 \pm 4.6	1.1 \pm 1.5	54.0
Station taking	1.7 \pm 4.1	0.4 \pm 0.7	63.0
Amplexus	0.7 \pm 1.6	0.2 \pm 0.5	67.5