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How and to what extent do sneakers gain proximity to females in an externally fertilizing fish?

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Recent studies have shown that fertilization success in external fertilization systems is influenced by differences in the temporal and spatial positions of competing males at ejaculation, as is the case for internal fertilization systems. Little is known about how these positions differ between competitors. We tackled this issue in the triplefin fish *Enneapterygius etheostoma*, which spawns in turbulent flow environments, and which employs external fertilization and alternative reproductive tactics (i.e. sneakers and territorial males). A frame-by-frame analysis of video images of sneaking behaviours recorded in the wild revealed that sneakers ejaculated later, and further from the spawning female, than territorial males. The majority of sneaking attempts failed because of attacks by territorial males. Successful sneakers mostly hid behind shelters around the spawning sites and then rushed into the site (i.e. were undetected), or were detected and attacked as they approached the site or hid near it, but rushed in anyway (attack-initiated sneaking). Of the undetected successful sneakers, a minority rushed in when the spawning female was being ignored by a territorial male that was attacking other sneakers (induced sneaking) and the others rushed in when there was no territorial attack (attack-free sneaking). Attack-free sneaking was carried out by sneakers that hid closer to the spawning female for longer, and were spatially and temporally closer to territorial males at ejaculation. Altogether, we conclude that the abilities to get past territorial vigilance and observe gamete release of spawning pairs at a hiding place are key factors that helped sneakers to improve their position at ejaculation, and thus perhaps their fertilization success.

Alternative reproductive tactics (ARTs) are one of the within-species discontinuous variations, and are particularly widespread in teleost fishes in which males typically employ either 'territorial' or 'sneaky' tactics (Oliveira, Taborsky, & Brockmann, 2008). The two tactics are often subject to different selection pressures, which causes the discontinuous variation of phenotypic traits between them. Territorial males monopolize primary access to females by defending critical resources that attract them and often invest in sexual behavioural displays (e.g. courtship, contest). Therefore, selective pressures on these males favour these behaviours and the necessary traits required for them (e.g. nuptial coloration, weaponry). In contrast, sneaker males exploit the reproductive effort of territorial males by attempting to steal fertilizations. Selective pressures on these males favour investment in behavioural, morphological and physiological traits that enable them to steal fertilizations (e.g. cryptic appearance, increased testes investment). The intrasexual variation in these species offers an ideal system in which to study general issues in evolutionary and behavioural ecology (e.g. evolution and functional causes of phenotypic variation; Oliveira et al., 2008).

Mating in species with ARTs is characterized by sperm competition, in which sperm from different males compete for the same ova. Adaptation to sperm competition has been intensively studied. How sperm compete (i.e. sperm competition mechanisms) is a critical aspect of male adaptations to sperm competition and is largely influenced by the mode of fertilization. In an internal fertilization system, two or more males cannot simultaneously copulate with a female. Therefore, sperm competition obeys the loaded raffle principle, in which mating order affects fertilization success (Birkhead & Hunter, 1990; Parker, 1998; see also Evans & Magurran, 2001, for a fish example). In external fertilization, which is predominant in teleosts, several males may ejaculate simultaneously, and there is a narrow window for fertilization because shed gametes may be diffused swiftly by turbulent water currents (Levitan & Petersen, 1995; Petersen, Warner, Cohen, Hess, & Sewell, 1992). Consequently, the importance of mating order is less emphasized in this fertilization mode. In externally fertilizing

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fish with ARTs, however, males competing for the same ova (i.e. territorial males and sneakers) do not necessarily experience simultaneous ejaculation, and mating order possibly arises between them as a function of differences in their initial positions before ejaculation (e.g. nest entry order, Berejikian et al., 2010; Blanchfield, Ridgway, & Wilson, 2003; Watanabe, Takamura, & Maekawa, 2008). A growing body of evidence, mainly in salmonids, shows that in this fertilization mode, the mating order, if assigned experimentally, strongly influences fertilization success under sperm competition. In vitro fertilization experiments, for example, show that first males have fertilization precedence, even if the timing differs only slightly between competitors (Gile & Ferguson, 1995; Hoysak, Liley, & Taylor, 2004; Mjjølnerod, Fleming, Refseth, & Hindar, 1998; Yeates, Searle, Ward, & Gage, 2007). The evidence clearly shows that in order to understand sperm competition selection and male adaptations, it is important to clarify the underlying dynamics and interactions of behaviours, gamete encounters and fertilization (Fu, Neff, & Gross, 2001; Stoltz & Neff, 2006b). To date, only a handful of studies have explored this issue (Sørum, Figenschou, Rudolfsen, & Folstad, 2011; Stoltz & Neff, 2006a).

In this study, we addressed this issue by examining how and to what extent competing males obtain temporal and spatial proximity to spawning females at the time of ejaculation in an externally fertilizing triplefin fish, *Enneapterygius etheostoma* (Tripterygiidae). This fish inhabits the rocky littoral zone of East Asian coastal waters. Male *E. etheostoma* exhibit ARTs whereby they switch from sneaking to territorial tactics as they grow (Hamada & Nakazono, 1989; H. Nonogaki, personal observation). Both sexes have markings in the form of six or seven dark brown bars, against a creamy/whitish ground body colour. During the breeding season, each territorial male defends a small area with a 5–7 cm radius that contains red algae (Rhodophyta) used as a spawning substrate. The territorial males also express melanistic nuptial coloration and dance in front of the first approaching female. Although such conspicuous colour and behaviour disappear once spawning begins, females may participate in ongoing spawning, often mating polygynously (one to seven females spawning simultaneously). Females produce 100–500 eggs per clutch, with an interval of several days between clutches (Shiogaki & Dotsu, 1973b), and deposit the entire clutch in a spawning site (i.e. with a single territorial male; Hamada & Nakazono, 1989; Shiogaki & Dotsu, 1973b). Because a female lays eggs among the algae at approximately 30 s intervals, its spawning episode lasts approximately 1 h. During spawning periods, the territorial male usually stays close to the spawning female and carefully observes it for sign of gamete release (i.e. body quivers, Shiogaki & Dotsu, 1973b). In response to each egg deposition, the male approaches the female, turns its body, and ejaculates while alongside the female, in order to synchronize gamete release (Hamada & Nakazono, 1989; Shiogaki & Dotsu, 1973b; see Supplementary video 1). Smaller sneaker males do not express the nuptial colour and therefore remain cryptic within their habitats; they may thus be better able to steal fertilizations by means of sneaking.

Around each defended territory at which spawning takes place, the territorial male is highly aggressive to sneakers, which keeps them further away from the spawning female than the territorial male. Sneakers hide behind arbitrary shelters to avoid being attacked and excluded by the territorial male. Sneakers may rush into the spawning sites after directly observing the quivering of the female's body that indicates egg deposition. However, body quivers are relatively inconspicuous and eggs are small (ca. 1 mm in diameter) and reddish but transparent (invisible to human observers, Hamada & Nakazono, 1989; Shiogaki & Dotsu, 1973a). Alternatively, sneakers may detect egg deposition by 'eavesdropping' on the conspicuous ejaculation behaviour of territorial males. All else being equal, the abilities of a sneaker to monitor the activities of a spawning pair and recognize signs of gamete release would be a function of the distance to the spawning pair (see Gross, 1991), and the delay of ejaculation from territorial males would simply increase with the distance of the rush into the spawning site. The spatial and temporal differences between the sneaker's and the territorial male's positions at ejaculation are therefore expected to increase with the distance. In other words, the abilities of a sneaker to monitor spawning and recognize signs of gamete release would be key factors that help sneakers to improve their position at ejaculation. In this study, we noted the behaviours of sneakers before sneaking (hiding) and the effects on the positions that sneakers achieved at ejaculation.

METHODS

Field Survey

Field work was conducted from early June to early July 2014, at Morode Beach (33°00′N, 132°50′E) on the west coast of Shikoku Island, Japan. At this location, *E. etheostoma* is found exclusively in the subtidal rocky zone (<2 m deep) where wave action generates flowing and turbulent environments. This species was reproductively active during the study period and spawning occurred on most mornings (Ota, Nonogaki, & Kohda, n.d.).

We visited the subtidal rocky area every morning (0700–1000 hours), and looked for *E. etheostoma* spawning events. When an event was discovered, we mounted a water-proof video camera (GZ-RX130, JVC–KENWOOD Co., Kanagawa, Japan) on a tripod at a given distance in order to obtain a close-up image of the entire spawning site (male territory; i.e. an image with radius 5–7 cm). The camera was set in an upright position to provide an overhead view because this fish species spawns on the vertical surface of rocks. The observed sites were tagged to avoid replicating observations. We located 41 different spawning sites and recorded each spawning event for 15 min. We also recorded a scene in which a ruler was placed along the rock surface at each spawning site to calibrate the distance for the following analyses. One to four females spawned simultaneously in each event (mean ± SD = 1.6 ± 0.8).

Recorded videos were viewed on a computer screen and analysed frame by frame, to determine the temporal and spatial positions of sneakers relative to territorial males at each ejaculation event. Of the 41 recordings, five were removed from analysis because spawning was disturbed for several minutes by attacks from the predatory stingfish, *Sebastiscus marmoratus* ($N = 2$), because spawning was shielded several times by schools of cardinal fish, *Apogon notatus* ($N = 1$), or because the video quality was poor due to insufficient light ($N = 2$). We therefore analysed 36 spawning events. The moment at which a territorial male was closest to a spawning female during ejaculation (i.e. the moment at which it turned alongside the female) was considered the moment of its ejaculation ($N = 1485$), and the time of this event was noted to the nearest 0.03 s. The closest proximity of the genital papilla of the spawning pair at each ejaculation moment was measured to the nearest 0.1 pixels and was considered its spatial position at ejaculation ($N = 1431$; 54 observations were unreliable and thus omitted). Although the overhead view did not allow us to identify its exact position, the fifth ray of the second dorsal fin was considered the position of the genital papilla. We were not able to obtain data for territorial males in 16 cases in which their ejaculations were displaced by sneakers thrusting themselves between the spawning pair.