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# Comparative responses of ovipositing *Anopheles gambiae* and *Culex quinquefasciatus* females to the presence of *Culex* egg rafts and larvae

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**Abstract.** Field observations have demonstrated that gravid *Anopheles gambiae* Giles *s.s.* (Diptera: Culicidae) are selective in their choice of oviposition sites. For example, immature stages of *An. gambiae s.s.* are rarely found in water that contains *Culex quinquefasciatus* Say immatures. The possibility that this may, in part at least, reflect a response by ovipositing *An. gambiae s.s.* females to volatile signals associated with *Culex* juveniles was evaluated by testing the response of *An. gambiae s.s.* females to varying densities of *Cx. quinquefasciatus* egg rafts and/or larvae in oviposition choice assays. For comparison, the oviposition choices of *Cx. quinquefasciatus* to conspecific egg rafts and/or larvae were similarly assayed. At a low density of *Cx. quinquefasciatus* egg rafts (1–15 egg rafts/100 mL water), *An. gambiae s.s.* females laid more eggs in the treatment water than in the control, with a maximum of twice as many in the treatment water at 5 egg rafts/100 mL water. At higher egg raft densities and in all treatments that included *Cx. quinquefasciatus* larvae, oviposition decreased significantly in the treatment dishes in a density-dependent manner. As previous studies have indicated, ovipositing *Cx. quinquefasciatus* females were attracted to and laid egg rafts in dishes containing conspecific egg rafts and, interestingly, also in dishes containing larvae.

**Key words.** *Anopheles gambiae s.s.*, *Culex quinquefasciatus*, attraction, avoidance, egg rafts, larvae, oviposition.

## Introduction

Mosquitoes spend the first part of their lifecycle in aquatic habitats and therefore their choice of appropriate oviposition sites has significant bearing on maternal reproductive success (Millar *et al*., 1994). Several attributes of the oviposition site influence hatching success and larval survival, including potential levels of predation and competition from other species, and high densities of conspecifics (Resetarits & Wilbur, 1989; Rejmánková *et al*., 1996; Onyabe & Roitberg, 1997; Angelon & Petranka, 2002; Kiflawi *et al*., 2003; Mokany & Shine, 2003; Blaustein *et al*., 2004; Sumba *et al*., 2008). Thus, females can be expected to be highly specific in choosing oviposition sites (Petranka & Fakhourry, 1991). The possibility that this may be mediated by semiochemicals has been widely recognized (Allan *et al*., 1987; Beehler *et al*., 1992). Chemical cues can originate from natural water bodies as breakdown products of bacterial origin (Sumba *et al*., 2004), from the mosquito as oviposition pheromones (Bentley & Day, 1989; Sumba *et al*., 2008), and from competitors or predators as allomones (Petranka & Fakhourry, 1991; Beehler *et al*., 1994; Spencer *et al*., 2002). The different sources of stimuli result in patterns

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of distribution of immature-stage mosquitoes that reflect differences in the suitability of sites for the development of different species (McCall & Cameron, 1995; Sumba *et al*., 2008).

Previous field observations have documented striking differences in the typical breeding sites of *Anopheles gambiae* and *Culex quinquefasciatus.* Gravid *Culex* spp., including *Cx. quinquefasciatus*, are preferentially attracted to relatively large, longer-lasting, aqueous habitats with high organic contents, such as soakage pits and hay and grass infusions (Kramer & Mulla, 1979; Millar *et al*., 1992, 1994; Takken & Knols, 1999). By contrast, gravid *An. gambiae* prefer to oviposit mainly in fresh, relatively small and transient water pools with sparse vegetation and little organic matter (Service, 1977; Minakawa *et al*., 1999, 2005a, 2005b; Gimning *et al*., 2001). Recent field surveys of larval habitats at three sites along the Kenyan coast (C. Mbogo, personal communication, 2004) and around Lake Victoria (Sumba, 2004) showed gradation of habitat types, with no clear spatial differentiation, and the presence of both *An. gambiae* and *Cx. quinquefasciatus.* In some ponds, *An. gambiae* were found to precede *Cx. quinquefasciatus* and the simultaneous occurrence of larvae of the two mosquito species in an individual pond was relatively infrequent. These observations suggest that when a habitat is suitable for oviposition by either species, one species may actively avoid it if it is already occupied by juveniles of the other. The specific objective of the present study was to examine how the oviposition selection behaviour of *An. gambiae s.s.* was affected by volatile emissions associated with different densities of eggs and/or larvae of *Cx. quinquefasciatus.* For comparison, the oviposition choices of *Cx. quinquefasciatus* to conspecific egg rafts or larvae were similarly assayed.

## Materials and methods

### *Experimental mosquitoes*

Mosquitoes used for the experiments were obtained from established laboratory-reared colonies of *An. gambiae s.s*. (Mbita strain) at the International Centre of Insect Physiology and Ecology (ICIPE) Duduville Campus, Nairobi, Kenya. The strain was initially collected from anopheline pools at Mbita Point, Suba District, in western Kenya (November 2006). Larvae were reared in plastic trays ($39 \times 28 \times 14$ cm) in an insectary at a density of about 500 larvae per 3 L of distilled water. The rearing room was maintained at $32 \pm 2$ °C, 52% relative humidity (RH). The larvae were fed daily on Tetramin® fish food. The adult mosquitoes were kept in cubic cages ($30 \times 30 \times 30$ cm) in a separate room maintained at $26 \pm 2$ °C, 70–80% RH with a photoperiod of LD 12 : 12 h; light was provided by a fluorescent lamp. Male and female mosquitoes were kept in the same cages to allow for insemination. Mosquitoes were fed on 6% glucose solution *ad libitum*. Female mosquitoes aged 3–5 days were fed on blood from a volunteer's forearm for 10 min on 2 or 3 consecutive days. Approval for the use of human subjects was obtained from the Kenya National Ethical Review Board (protocol no. KEMRI/RES/7/3/1). Multiple bloodmeals have been shown to increase the chance of oviposition by females that are to lay their first batch of eggs (Briegel & Hörler, 1993).

A *Cx. quinquefasciatus* (Nairobi) colony was established in November 2006 and reared as outlined above for *An. gambiae s.s*. Female mosquitoes aged 3–5 days were fed on blood from an albino rat (*Rattus norvegicus*) for 10 min/day for 4 or 5 consecutive days prior to oviposition assays.

### *Oviposition water*

To simulate the microbial and chemical characteristics of the water in natural pools, water used in the bioassays was prepared as follows (Sumba *et al*., 2004): double-distilled water (20 L) was mixed with muddy soil (5 L collected from a pond previously colonized by eggs and larvae of *An. gambiae s.s* at Mbita Point, Suba District, western Kenya) and allowed to settle for 3–7 days. The supernatant from the mixture was then used in all oviposition bioassays and is referred to henceforth as 'pool water'.

### *Oviposition bioassays*

To investigate the role of olfactory cues in the oviposition behaviour of *An. gambiae s.s.*, a 'double-cup' oviposition set-up (Fig. 1), previously developed at ICIPE (Sumba *et al*., 2004), was used. This set-up consisted of an outer black plastic cup (8 cm deep, 6 cm diameter) containing 100 mL of the test substrate (i.e. pool water plus a known number of *Cx. quinquefasciatus* larvae and/or egg rafts) and a smaller, inner opaque plastic cup (2 cm deep, 4 cm diameter) containing 15 mL of only pool water. The inner cup floated in the pool water of the outer cup. A 7-cm diameter cone of filter paper (Whatman No. 1) was placed so that its tip dipped into the pool water in the inner cup and its sides rested on the top edges of the inner and outer cups (Fig. 1). This arrangement prevented mosquitoes from coming into direct tarsal or visual contact with the test substrate in the outer cup. As the tip of the cone dipped into the pool water in the inner cup, the rest of the cone rapidly became moist by capillary action.

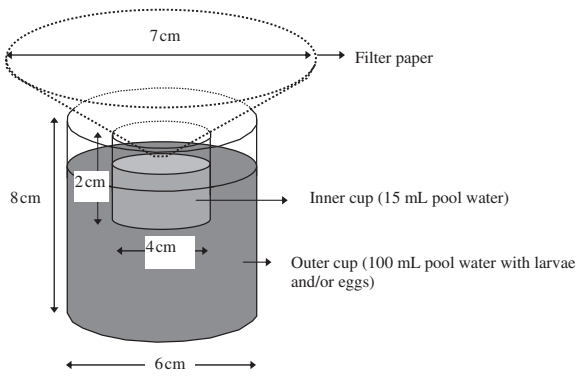


**Fig. 1.** The double-cup set-up used to prevent mosquitoes touching test substrates prior to or during oviposition (see text for details).

Odours from the test substrate in the outer cup were assumed to permeate the moist filter paper of the cone and to reach the female mosquitoes above.

Five-day-old gravid females in groups of 20 were released into the experimental cages (30 × 30 × 30 cm) at 15.30 hours and left to acclimatize for 1 h. Two double-cup oviposition set-ups, one with pool water containing *Cx. quinquefasciatus* egg rafts and/or larvae in the outer cup and the other with only pool water in the outer cup, were then introduced into the cages, positioned at diagonally opposite corners. The set-ups were removed 14 h later and the filter papers were withdrawn, examined under a dissection microscope and the number of eggs (for *An. gambiae*) or egg rafts (for *Cx. quinquefasciatus*) oviposited were counted. Three replicates were performed on each of 5 days for each density of each treatment. Fresh gravid female mosquitoes and oviposition substrates were used for each of the 15 replicates.

### *Response of* An. gambiae s.s. *to hetero-specific egg rafts and larvae*

The oviposition choices made by gravid *An. gambiae s.s.* females were investigated in response to three treatments over a range of densities: (a) *Cx. quinquefasciatus* egg rafts (1, 5, 10, 15, 20, 25, 50 or 100 rafts/100 mL pool water); (b) *Cx. quinquefasciatus* second-instar larvae (1, 5, 10, 15, 20, 25, 50 or 100 larvae/100 mL pool water), and (c) *Cx. quinquefasciatus* egg rafts (1, 5, 10, 15, 20, 25, 50 or 100 rafts/100 mL pool water) plus 10 second-instar larvae. Control set-ups contained pool water only.

### *Response of* Cx. quinquefasciatus *to conspecific egg rafts and larvae*

For comparison with *An. gambiae s.s.*, the same oviposition assays were conducted with *Cx. quinquefasciatus* females for two of the *An. gambiae s.s.* treatments: (a) *Cx. quinquefasciatus* egg rafts (1, 5, 10, 15, 20, 25, 50 or 100 rafts/100 mL pool water), and (b) *Cx. quinquefasciatus* second-instar larvae (1, 5, 10, 15, 20, 25, 50 or 100 larvae/100 mL pool water).

### *Data analyses*

An oviposition activity index (OAI) was calculated for each replicate in the dual choice assays according to the formula described by Kramer & Mulla (1979):

$$OAI = (N_t - N_c)/(N_t + N_c),$$

where, $N_t$ = the number of eggs on the test substrate (pool water with larvae and/or eggs) and $N_c$ = the number of eggs on the control substrate (pool water only). The significance of the OAI for each treatment was determined by one-sample Student's *t*-test. Oviposition activity index values can fall between +1 and −1. Positive numbers indicate that more eggs were laid on the test substrate than on the control substrate, whereas negative numbers indicate that more eggs were laid on the control substrate. According to Hwang (1980) and Poonam *et al*. (2002), a substance is considered to be active when the OAI is $\geq +0.3$ (i.e. more than twice as many eggs are laid on it as on the control) and repellent or deterrent when the OAI value is $\leq -0.3$. Analyses were carried out using SAS Version 9.1 (SAS Institute, Inc., Cary, NC, U.S.A.) and dose–response regressions. Means were compared by Student–Newman–Keuls (SNK) test.

## Results

### *Response of* An. gambiae *s.s. to hetero-specific egg rafts*

In the cage bioassay there was no positional bias (OAI = 0.02) for oviposition by gravid *An. gambiae s.s.* when pool water was presented simultaneously in both double-cup set-ups (i.e. no egg rafts/100 mL pool water). When *Cx. quinquefasciatus* egg rafts were added to the pool water (Fig. 2A), the OAI was positive for the four lowest densities (1, 5, 10 and 15 rafts/100 mL) and, although none of the means were significantly different from the control (0 rafts/100 mL) for this group of densities, there was a maximum OAI > 0.3 at a density of 5 egg rafts/100 mL, which means that >67% of eggs were laid in the treatment cups. At higher densities (≥25 rafts/100 mL) the OAI values were negative and differed significantly from the lower densities ($P < 0.05$, *t*-tests). At these higher densities, the mean OAI was $< -0.3$ (i.e. <33% of eggs were laid in the treatment cups) (Fig. 2A). This indicates that *Cx. quinquefasciatus* egg rafts were attractive to ovipositing *An. gambiae s.s.* females at a density of 5 egg rafts/100 mL and were significantly repellent at densities ≥25 egg rafts/100 mL.

### *Response of* An. gambiae s.s. *to hetero-specific larvae*

Gravid females of *An. gambiae s.s.* laid increasingly lower proportions of their eggs in the treatment oviposition cups in response to increasing densities of larvae, reaching a minimum that levelled off at a density of 50 larvae/100 mL (OAI = −0.46). Oviposition activity index values at ≥50 larvae were statistically significantly different from control values ($P < 0.05$, *t*-statistics (Fig. 2B). Hence, *Cx. quinquefasciatus* larvae had no significant effect on the distribution of *An. gambiae s.s.* eggs at densities of ≤25 larvae/100 mL, but were significantly deterrent at densities ≥50 larvae/100 mL.

### *Response of* An. gambiae s.s. *to hetero-specific egg rafts plus larvae*

The proportions of eggs deposited by *An. gambiae s.s.* females in the treatment cups were significantly lower than those laid in the control cups ($P < 0.05$, *t*-statistics) for all

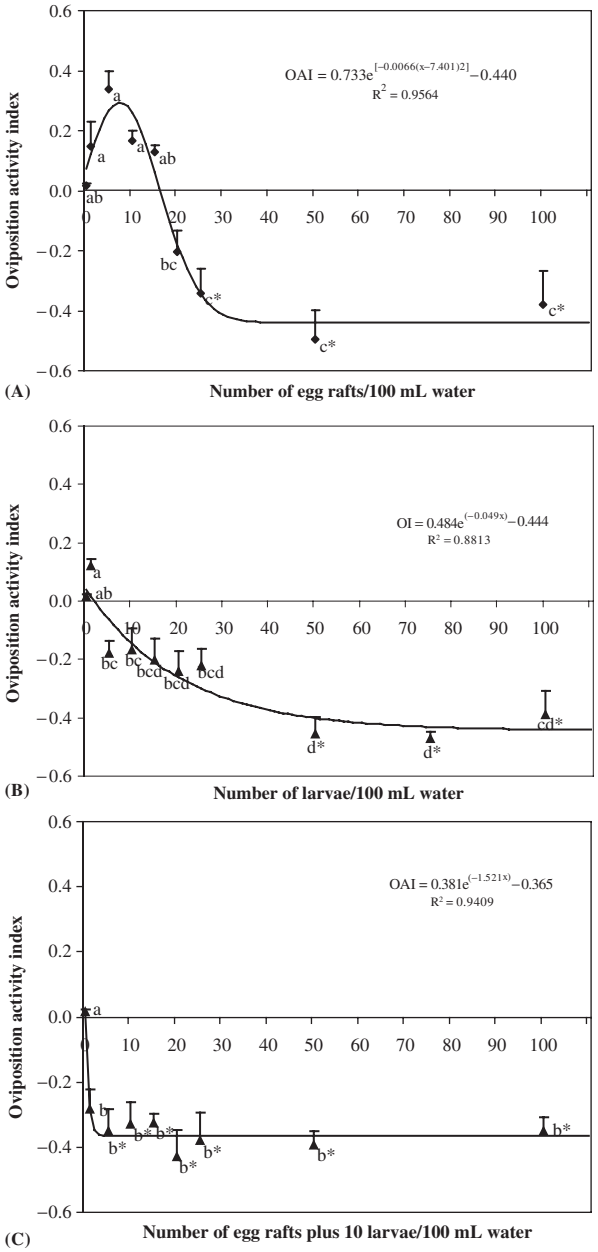


**Fig. 2.** *Anopheles gambiae s.s.* response to different densities of *Culex quinquefasciatus* egg rafts and/or larvae as measured by the oviposition activity index (OAI) (means ± standard error; $n = 15$ assays for each treatment), with regression relationship. (A) Egg rafts at densities as indicated. (B) Second-instar larvae at densities as indicated. (C) Egg rafts at densities as indicated plus 10 second-instar larvae in pool water vs. a control of pool water only. $^{*}P < 0.05$ significantly differs from zero ($t$-test); OAIs with different letters at different doses differ significantly from one another (Student–Newman–Keuls test, $P < 0.05$).

densities of egg rafts plus larvae (Fig. 2C), with a mean OAI across all densities of $<-0.3$. Hence, the presence of even one egg raft/100 mL water increased the deterrent effect of 10 larvae/100 mL to that of ~30 larvae/100 mL. Oviposition

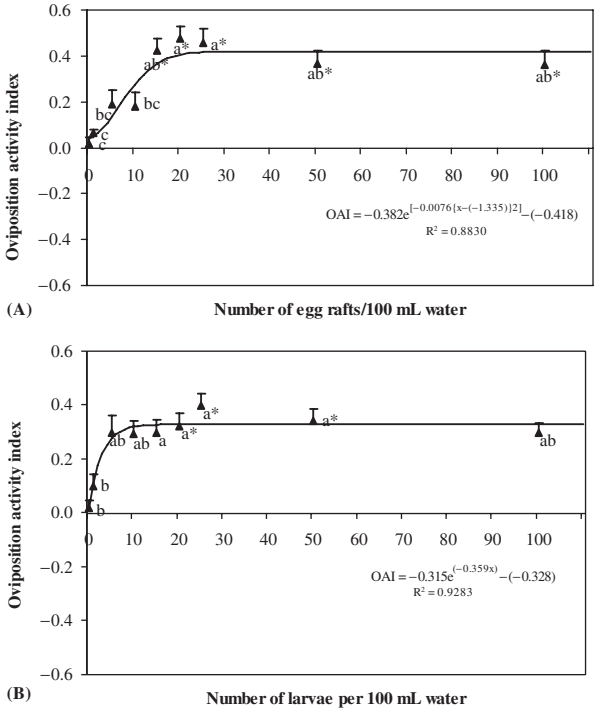


**Fig. 3.** *Culex quinquefasciatus* response to different densities of conspecific egg rafts and/or larvae as measured by the oviposition activity index (OAI) (means ± standard error; $n = 15$ assays for each treatment), with regression relationship. (A) Egg rafts at densities as indicated. (B) Second-instar larvae at densities as indicated in pool water vs. a control of pool water only. $^{*}P < 0.05$ significantly differs from zero ($t$-test); OAIs with different letters at different doses differ significantly from one another (Student–Newman–Keuls test, $P < 0.05$).

activity index values for 10 larvae in the presence (−0.35) and absence (−0.17) of five rafts were found to differ significantly ($P < 0.05$, $t$-statistics).

### *Response of* Cx. quinquefasciatus *to conspecific egg rafts and larvae*

As expected, the presence of conspecific egg rafts caused significant increases in the proportions of *Cx. quinquefasciatus* egg rafts laid in the treatment cups, reaching a maximum OAI at ~25 egg rafts/100 mL (Fig. 3A). Interestingly, conspecific larvae induced similar increases in OAI values, with a density–response pattern comparable with that for egg rafts (Fig. 3B). Overall, the OAI was ≥0.3 for densities of ≥20 egg rafts/100 mL or ≥5 larvae/100 mL.

## Discussion

Our results show that *An. gambiae s.s.* females exhibit varying oviposition response patterns depending on the density of larvae and/or eggs of *Cx. quinquefasciatus* present in the water. When the densities of *Cx. quinquefasciatus* egg

rafts present in the treatment double-cups were reduced to ~5 egg rafts/100 mL, the OAI was ~0.3, indicating that *An. gambiae s.s.* females laid twice as many eggs in the treatment cups as in the control cups. These results must be treated with caution, however, because the mean OAIs did not differ statistically significantly from those for the control. At densities of ≥25 egg rafts/100 mL, however, *An. gambiae s.s.* females were statistically significantly deterred from ovipositing in the treatment containers (Fig. 2A). The presence of *Cx. quinquefasciatus* larvae deterred oviposition by *An. gambiae s.s.* females at all densities tested, and statistically significantly so at densities of ≥50 larvae/100 mL (Fig. 2B). The deterrence effect of 10 larvae/100 mL was enhanced roughly three-fold by adding one or more egg rafts (Fig. 2B, C).

The attraction of *An. gambiae s.s.* to low densities of *Cx. quinquefasciatus* egg rafts may account for some co-presence of the two species in the field (C. Mbogo, personal communication, 2004; Sumba, 2004). However, the general pattern of our laboratory results indicates that *An. gambiae s.s.* actively avoids ovipositing in pools already occupied by *Cx. quinquefasciatus* juveniles. As the double-cup oviposition set-up did not allow mechanical, chemo-tactile or visual contact with eggs or larvae, the avoidance by *An. gambiae s.s.* of water containing *Culex* juveniles appears to be mediated by volatile olfactory signals.

An interesting question concerns whether the observed effects of the presence of *Culex* egg rafts and larvae are caused by the same or different chemical signals. The deterrent effect of 10 larvae/100 mL appears to have been dramatically enhanced by the addition of even a single egg raft, which is contrary to the finding that low densities of egg rafts are associated with positive OAIs. Thus, it is tempting to speculate that the mode of action of volatiles from egg rafts differs from that of volatiles from larvae. If we assume there is a simple additive effect of the same signal from egg rafts and larvae, the addition of five egg rafts and 10 larvae to 100 mL of water would be expected to result in a small positive OAI. The negative OAI of $<-0.35$ (Fig. 2C) suggests a synergistic interaction between two different sets of signals.

Detailed investigations of the chemistry of volatiles associated with the eggs and larvae of *Cx. quinquefasciatus* and their dose–response effects, separately and in combination, on the oviposition behaviour of *An. gambiae*, will help to shed further light on this issue.

Previous studies designed to clarify the types of signals that mediate the attraction of *An. gambiae* to specific pools demonstrated the mediation of several signals (Sumba *et al*., 2004, 2008). Volatiles associated with the microbial population in preferred anopheline pools were found to be important initial inter-specific attractants (Sumba *et al*., 2004). In addition, two intraspecific signals were shown to regulate oviposition: a volatile pheromone and a contact deterrent associated with the larval stages of the mosquito (Sumba *et al*., 2008). This pair of signals may play an important role in fine-tuning the balance between the exploitation of a suitable breeding site and the avoidance of intraspecific competition and other effects of overcrowding (Gimning *et al*., 2002; Koenraadt & Takken, 2003; Kiflawi *et al*., 2003; Sumba *et al*., 2008). The present study adds another dimension to our understanding of the complex set of signals used by *An. gambiae* to locate preferred oviposition habitats. Our results suggest that, whereas such habitats exert a 'pulling' effect, unsuitable pools may exert an active 'pushing' effect, and it is the integrated 'push/pull' effects of these different signals that may effectively guide an insect to a habitat suitable for its species. Previous observations in two different agro-ecological zones in Kenya (C. Mbogo, personal communication, 2004; Sumba, 2004) appear to be consistent with our laboratory results. However, more detailed field surveys are needed to confirm the role of *Culex* volatiles in actively 'pushing' *An. gambiae* away from pools favoured by *Culex* spp. and, thus, guiding it towards more suitable habitats.

The oviposition responses of *Cx. quinquefasciatus* to conspecific egg rafts and larvae were included in the present study for comparison with those of *An. gambiae s.s*. As previous studies (Kramer & Mulla, 1979; Millar *et al*., 1992, 1994; Takken & Knols, 1999) led us to expect, the presence of conspecific egg rafts caused density-dependent increases in oviposition, reaching an optimum at a certain egg density (Fig. 3A). This is probably mainly the effect of the well-studied oviposition pheromone (5*R*,6*S*)-6-acetoxy-5-hexadecanolide associated with the egg rafts (Laurence & Pickett, 1982; Mboera *et al*., 2000; Olagbemiro *et al*., 2004). A similar effect of volatiles associated with larvae (Fig. 3B) has not been reported previously. Our results suggest the need for further studies on *Culex* larvae and their pheromonal signal(s) with regard to oviposition to establish whether they resemble or differ from that of the egg pheromone and, if the larval pheromone differs, to discover how the two interact to regulate the oviposition behaviours of these mosquito species.

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