

## NOTES

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### No ecological relevance of trimethylamine in fish—*Daphnia* interactions

**Abstract**—Diel vertical migration (DVM) is a widespread predator-avoidance behavior in *Daphnia* and can be induced by kairomones released by fish. It has recently been shown in laboratory experiments that DVM in *Daphnia hyalina* can be induced by micromolar concentrations of trimethylamine (TMA). The authors concluded, after spectrophotometrical determination of the amine content in fish incubation water, that TMA is an active component of the kairomone. Using a selective solid phase microextraction (SPME)/GCMS method, we show that the TMA content in fish incubation water was overestimated by several orders of magnitude. The amounts of TMA released by any of the three cyprinids *Leuciscus idus*, *Leucaspis delineatus*, and *Carassius carassius* have no relevance for DVM induction and TMA concentrations detected in fish incubation water did not induce DVM. Further, we show that efficient removal of existing TMA traces from fish incubation water did not decrease the kairomone activity. This excludes TMA as part of a kairomone cocktail. Hence TMA must not be called a kairomone in the fish-*Daphnia* context.

Diel vertical migration (DVM) is a widespread strategy employed by marine and freshwater zooplankton to reduce predation risk by migration to greater depth during day and thus to minimize the chance of being detected by visually orientating predators like fish (Zaret and Suffern 1976; Stich and Lampert 1981). The adaptive response of DVM can be chemically induced in *Daphnia* by predator-associated chemical cues (kairomones) (Dodson 1988). To avoid maladaptive responses, predator-borne chemical cues have to reflect exclusively the predation risk and no other natural sources of the cue should be present (Von Elert and Pohnert 2000). This is crucial since DVM in stratified lakes has a high demographic cost due to low temperature and food availability in the hypolimnetic refuge. Indeed, the DVM-inducing kairomone seems to be a specific predictor of the risk of predation, as timing and amplitude of migration are closely related to fish abundance and kairomone concentration (Ringelberg et al. 1991; Loose 1993; Loose and Dawidowicz 1994; Von Elert and Pohnert 2000).

The chemical nature of the DVM-inducing kairomone from cyprinid fish has been preliminarily characterized as a low molecular weight compound that could be quantitatively enriched from fish incubation water with lipophilic C18 solid phase extraction (Von Elert and Loose 1996). The activity of the crude kairomone could be reversibly deactivated by acetylation with acetic anhydride. Amines are not likely to be a structural element of the kairomone, since it was not interacting with cation-exchangers. Similar features were reported for kairomones released by pike (*Esox lucius*, Esocidae) and stickleback (*Gasterosteus aculeatus*, Gasterosteidae)

suggesting that the kairomones have no, or low, fish species specificity (Von Elert and Pohnert 2000).

Based on the finding that the kairomone is, at least partially, released by bacteria associated with planktivorous fish (Ringelberg and van Gool 1998), Boriss et al. (1999) tested trimethylamine (TMA) as a possible candidate for the active factor. Bacteria in the skin of fish release TMA as a main metabolite of fish-borne trimethylamine-N-oxide (TMAO). TMAO is involved in osmoregulation of euryhaline and marine fish (Yancey and Somero 1979; Van Waarde 1988). Recently, Boriss et al. (1999) determined that concentrations of TMA >40  $\mu\text{M}$  induce DVM in *Daphnia*. In addition, an unspecific spectrophotometrical determination of amines in DVM-inducing fish incubation water showed a response equivalent to micromolar TMA concentrations. From these observations, the authors concluded that TMA is an active component of the DVM-inducing kairomone.

The striking discrepancies between the chemical properties of TMA and those reported from the preliminary characterization of the kairomone (Loose et al. 1993; Von Elert and Loose 1996) and the very high TMA concentrations, corresponding to >1.4 mg L<sup>-1</sup> dissolved organic carbon required for the induction of DVM (Boriss et al. 1999), led us to reinvestigate the ecological role of TMA as a kairomone.

**Trimethylamine determination**—To reinvestigate the TMA content in the experimental set-up used by Boriss et al. (1999) fish water was produced as described by incubating *Leuciscus idus* for 24 h at a density of 4 fish per 10 liters.

We determined the TMA content of this fish water after solid phase microextraction (SPME) using GC/MS detection. The extraction and quantification procedure of TMA was based on a protocol by Mills et al. (1999) and modified to increase sensitivity to the required threshold. For the detection of concentrations down to 1  $\mu\text{M}$  TMA, a 5-ml aliquot of freshly collected water from fish tanks was transferred directly to a 10-ml sample vial equipped with a glass mantled magnetic stirrer. Sodium hydroxide (0.5 g) was added to the sample and the vial was sealed immediately with a polytetrafluoroethylene septum cap. The solution was stirred in a 50°C water bath and the volatiles from the headspace were extracted immediately with a polydimethylsiloxane SPME fiber (100  $\mu\text{m}$  film thickness, SUPELCO, Deisenhofen, Germany) as described (Mills et al. 1999). The sample was directly desorbed in the hot (220°C) injector of a Varian 3400 GC (splitless mode, split closed for 1 min; He flow: 1 ml min<sup>-1</sup>; temperature program 35°C (4 min) ramped at 35°C min<sup>-1</sup> to 280°C (2 min); Optima 5 MS column, 30 m, Macherey Nagel, Düren). Detection of TMA ( $m/z = 59$  [M<sup>+</sup>,10],

Table 1. TMA content of fish incubation water determined by solid phase microextraction (SPME) and gas chromatography/mass spectrometry.

| Species                      | Incubation time (h) | Density (fish L <sup>-1</sup> ) | TMA content (nM) |
|------------------------------|---------------------|---------------------------------|------------------|
| <i>Leuciscus idus</i>        | 24                  | 0.4                             | <<10             |
| <i>Leuciscus idus</i>        | 24                  | 4                               | ca.5             |
| <i>Leucaspius delineatus</i> | 24                  | 1                               | <<10             |
| <i>Leucaspius delineatus</i> | 36                  | 2                               | 30               |
| <i>Carassius carassius</i>   | 24                  | 0.4                             | <<10             |

58[100], 42[34], 30[25]) with a Finnigan MAT Magnum mass spectrometer gave a linear response over the range of 1–100  $\mu\text{M}$ . To further increase the sensitivity, larger aliquots of water from fish tanks were acidified with HCl (6N, pH = 1) and concentrated in vacuum without loss of quantitatively formed TMA·HCl salt. After addition of 1.5 g of NaOH pellets (caution, heat of neutralization) to the ice cooled sample analysis could be performed as described above. Thus concentrating 500 ml of acidified fish water to 5 ml extraction volume before pretreatment of the sample with NaOH led to a 10 nM detection limit.

The TMA content in samples prepared as described by Boriss et al. (1999) was clearly below this threshold of detection (Table 1, entry 1) and a second set of samples, prepared by incubating a ten times higher number of *Leuciscus idus*, showed traces of TMA just below the linear threshold of detection (Table 1, entry 2). The TMA concentration per fish specimen and volume were more than four orders of

magnitude lower than reported by Boriss et al. (1999). Similar results were obtained with incubation water from different cyprinid species (Table 1, entries 3–5).

To make sure that our preparations were not inherently lower in TMA than those reported originally, we used the same unspecific spectrophotometric method as Boriss et al. (1999) for the determination of tertiary alkylamines (Kakác and Vejdelek 1974). In accordance with Boriss et al. (1999) we found a photometric response equivalent to the reported value of 10–25  $\mu\text{M}$  total tertiary amines for the preparation with 4 fish in 10 liters of water. However, since the spectrophotometric technique lacks specificity for the determination of definite tertiary alkylamines in samples of unknown composition (Kakác and Vejdelek 1974), the amounts of TMA in fish water reported by Boriss et al. (1999) were drastically overestimated.

**Bioassays**—In order to evaluate the ecological role of TMA in the induction of DVM by freshwater fish we used a clone of *Daphnia magna* in a bioassay system similar to that in the original report (Loose et al. 1993; Boriss et al. 1999). For each replicate five 2-day old individuals of the clone were placed in a perspex tube (90 cm long, 1.5 cm in diameter) filled with 0.45  $\mu\text{m}$  filtered, aged lake water (day : night cycle 18 : 6 h). The tubes were placed in a water bath with a temperature gradient from 20 to 10°C (Fig. 1d) and the mean day depth of the five daphnids per tube was determined after 3 days. Each treatment was run in replicates of 3 (except for *Leucaspius delineatus* with  $N = 5$ ) and the response to a treatment was calculated as the mean ( $\pm$  standard error) of the day time depths of the replicates. We ver-

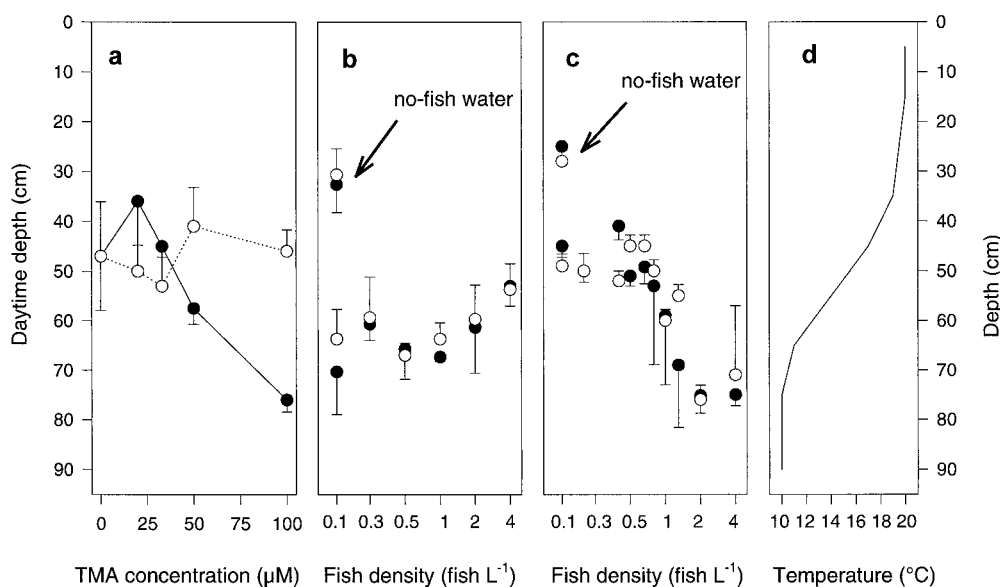


Fig. 1. Behavioral response of *D. magna* to trimethylamine and incubation water of two cyprinid species without (filled circles) and after TMA-removal by evaporation under strongly alkaline conditions (open circles). The response is given as mean daytime depth ( $\pm$  standard error) of each of five daphnids in replicate treatments. Maximum mean daytime depth (90 cm) indicates maximum biological activity. Effects on the mean daytime depth are shown for (a) different concentrations of TMA added to lake water ( $N = 3$ ). (b) the number of *L. idus* per liter ( $N = 3$ ). (c) the number of *L. delineatus* per liter ( $N = 5$ ). (d) Temperature profile of the vertical tubes used in the assay.

ified that the induction of DVM required the same threshold of 40–50  $\mu\text{M}$  TMA (Fig. 1a) as reported (Boriss et al. 1999), indicating that there was no difference in sensitivity of the clones used for the experiments.

For the purpose of comparison with the results of Boriss et al. (1999) we used the same fish species (*L. idus*) for further experiments. Fish incubation water was produced at a density of 4 fish  $\text{L}^{-1}$  and subsequently diluted with control water to 0.125 fish  $\text{L}^{-1}$ . Kairomone concentration in this water exceeded the threshold several fold, as is indicated by the strong biological activity observed after dilution of this water with non-fish water (Fig. 1b). Since biologically active water could be diluted 32-fold without a significant loss of activity, unrealistically high (1.28 mM) TMA concentrations should have been present in the undiluted sample if this compound were a biologically significant kairomone. However we could not determine TMA (detection limit 10 nM) in this highly biologically active fish incubation water. This suggests that the TMA concentration was at least  $10^5$  times below the threshold for a response by *D. magna*. Similar findings were obtained from a dilution series with water from *L. delineatus* (Fig. 1c) with TMA-levels well below 10 nM (Table 1).

Volatile tertiary alkylamines like TMA are removed from samples if evaporated to dryness under strongly alkaline conditions. When artificially added TMA is used as the only DVM-inducing cue in non-fish water, all biological activity is lost after evaporation under strongly alkaline conditions (Fig. 1a). We used this approach to determine the contribution of all putatively present volatile tertiary amines to the biological activity of fish incubation water of *L. idus*. After removal of these volatile components, neutralization and redilution of the sample with ultrapure water, biological activity was not reduced when compared with the activity of untreated incubation water (Fig. 1b). Two-way ANOVA, 3 replicates per treatment, with 7 fish densities and 2 levels of TMA removal as independent variables showed that there is a fish density effect on daytime depth of *Daphnia* ( $df_{6, 28}$ ;  $F = 4.29$ ;  $P < 0.005$ ). However, TMA-removal had no significant effect ( $df_{1, 28}$ ;  $F = 0.99$ ;  $P = 0.32$ ;  $\text{MS}_{\text{Error}} = 310$ ) on the behavior of *Daphnia* and neither did it interact with fish density ( $df_{6, 28}$ ;  $F = 0.21$ ;  $P = 0.97$ ) (Fig. 1b). Similar results were obtained with incubation water of *L. delineatus* (Fig. 1c). A two-way ANOVA (5 replicates per treatment) with 10 fish densities and 2 levels of TMA removal as independent variables and with mean day depth as dependent variable was calculated. Fish density had a significant effect on mean daytime depth of *Daphnia* ( $df_{9, 80}$ ;  $F = 5.34$ ;  $P < 0.005$ ). The interaction term (fish density by TMA removal) was not significant ( $df_{9, 80}$ ;  $F = 0.48$ ;  $P = 0.88$ ;  $\text{MS}_{\text{Error}} = 227$ ) and neither was the factor TMA-removal ( $df_{1, 80}$ ;  $F = 0.23$ ;  $P = 0.63$ ). The results indicate that neither TMA nor volatile tertiary alkylamines in general are of any significance for the induction of DVM by cyprinid fish.

These results are further supported by the fact that DVM-inducing activity of a tertiary amine as TMA is incompatible with the findings that the kairomone of the three cyprinid species investigated in this study did not interact with cation-exchangers and that the biological activity of a crude kairomone extract can be destroyed by acetylation with acetic

Table 2. Effect of lipophilic solid-phase extraction (C18-SPE) on the DVM-inducing activity. Incubation water: 2 crucian carp (*C. Carassius*) were incubated for 24 h in 10 liters. Maximum mean day depth (90 cm) indicates maximum biological activity ( $N = 5$ ).

| Treatment                           | Mean daytime depth (cm $\pm$ SE) |
|-------------------------------------|----------------------------------|
| Incubation water                    | 76 $\pm$ 4                       |
| Incubation water after C18-SPE      | 3 $\pm$ 2                        |
| 100 $\mu\text{M}$ TMA               | 76 $\pm$ 2                       |
| 100 $\mu\text{M}$ TMA after C18-SPE | 68 $\pm$ 8                       |

anhydride (Von Elert and Loose 1996). Another feature common to kairomones from cyprinid and noncyprinid species is that they can be removed quantitatively from the incubation water by lipophilic C18-solid phase extraction (SPE) (Von Elert and Pohnert 2000). Though the biological activity of incubation water of *Carassius carassius* was significantly reduced (*t*-test,  $P < 0.005$ ) after C18-SPE, no removal of biological activity of the eluate was observed after extraction of a 100  $\mu\text{M}$  TMA solution (*t*-test,  $P = 0.39$ ) (Table 2).

## Conclusions

The current study has demonstrated that no relevant concentrations of fish borne TMA can be expected in lakes where DVM is observed. This finding is in accord with the fact that freshwater fish have inverse osmoregulation, and only traces of TMA are detected in freshwater fish as compared to marine fish (Lange and Fugelli 1965; Hebard et al. 1982). Furthermore, TMA does not fulfill the critical criterion for an efficient kairomone, since it is not specific for the presence of freshwater fish and, thus, does not reflect the predation risk: the food source of *Daphnia*, freshwater algae, like, e.g., *Ochromonas* (Herrmann and Jüttner 1977), *Gomphonema parvulum*, or *Asterionella formosa* (Pohnert and Von Elert unpubl. data) also produce significant amounts of TMA.

Despite the fact that we confirm the findings that elevated (>40  $\mu\text{M}$ ) TMA concentrations induce DVM only nanomolar concentrations of TMA were present in highly concentrated fish incubation water. Thus TMA can not be considered as a fish-borne kairomone. We observed no alteration of DVM inducing efficiency after TMA removal, excluding the possibility that TMA or other volatile amines act synergistically as component of a kairomone blend. This has further implications, since the DVM-inducing properties of fish incubation water as well as the life-history changes inducing activity is retained by C18-SPE (Stibor 1995) indicating that a role of TMA in fish-mediated life-history effects can be denied.

Since sources other than fish can account for the natural TMA level in lakes, detailed investigations on the origin, concentration in the natural environment and ecological function of this amine are required. In all cases, one has to be especially careful to distinguish between a true ecological function and the observation of possible laboratory artifacts, since micromolar concentrations of any metabolite, like TMA, might cause changes in, e.g., pH or ionic strength of

the water, or changes in the susceptibility of the investigated species towards other (true) signaling compounds. It is likely that the induction of DVM by unrealistic TMA concentrations is limited to laboratory systems in which the compound is artificially introduced. Since we showed that (1) TMA is not released in relevant concentrations by planktivorous fish, and (2) traces of TMA in fish incubation water do not contribute to its biological activity; TMA must not be called a kairomone in the fish–*Daphnia* context.

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