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The sex specific metabolic footprint of *Oithona davisae*

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ABSTRACT

In pelagic copepods, the group representing the highest animal abundances on earth, males and females have distinct morphological and behavioural differences. In several species female pheromones are known to facilitate the mate finding process, and copepod exudates induce changes in physiology and behaviour in several phytoplankton species. Here we tested whether the sexual dimorphism in morphology and behaviour is mirrored in the exudate composition of males and females. We find differences in the exudate composition, with females seemingly producing more compounds. While we were able to remove the sex pheromones from the water by filtration through reverse phase solid phase extraction columns, we were not able to recover the active pheromone from the solid phase.

1. Introduction

Copepods are small crustaceans that inhabit a wide range of habitats ranging from small waterbodies in plants, to groundwater reservoirs and the open ocean. Although they usually escape the human eye due to their small size and transparency, they may represent the animal class with the largest abundances on Earth (Humes, 1994). Pelagic copepods are a crucial link between primary production and higher trophic levels (Naganuma, 1996). As key component of marine food webs, copepods serve either directly or indirectly as food sources for most commercially important fish species and in recent years their importance as live feed is also increasing in intensive aquaculture (Drillet et al., 2011).

Copepods have a complex lifecycle and typically go through six naupliar and five copepodite stages before becoming sexually mature adults. Once adult, most species show distinct morphological and behavioural differences between males and females (Maly and Maly, 1999; Ohtsuka and Huys, 2001). Females are normally bigger than males and have a different body shape and number of urosome segments. They also have sexually dimorphic antennules, with pelagic males typically having either one or two geniculated antennules that allows them to grasp females by folding the antennule(s) during the mating process (Boxshall and Huys, 1998). In several pelagic species an additional doubling in the number of aesthetascs (chemical sensilla) on the antennules of males occurs during the final moult (Boxshall and Huys, 1998; Ohtsuka and Huys, 2001). These morphological differences are also accompanied by distinct swimming behaviours of the sexes. Adult copepod males mostly engage more in energetically costly mate searching behaviour, while females have a more passive role and concentrate on feeding. In some species males cease foraging completely and spend their short adult life searching for potential mating partners (Yen, 1988). Their mate search efforts are aided in several species by females producing pheromone trails that are followed by the males (i.e. Doall et al., 1998; Bagøien and Kiørboe, 2005). These trails can be relatively long compared to the size of the animals and their three-dimensional shape depends on the female's swimming pattern and the amount of released pheromones. While cruising copepods produce a continuous trail, the trail of an ambush feeding copepod that occasionally repositions itself by jumps will resemble a series of unconnected pheromone patches (Kiørboe, 2008; Heuschele and Selander, 2014).

In the cyclopoid copepod *Oithona davisae* males search for females following such a patchy pheromone "trail" (Uchima and Murano, 1988). However the presence of even low concentrations of female scent triggers a distinct search pattern that is characterized by faster swimming speeds and a more convoluted swimming path (Heuschele and Kiørboe, 2012) (Fig. 1A). In this species females can also distinguish between the scent of mated and virgin females, and adjust their search behaviour accordingly. This ability has also been shown in *Diaptomus leptopus* (Leeuwen and Maly, 1991). Besides information about species

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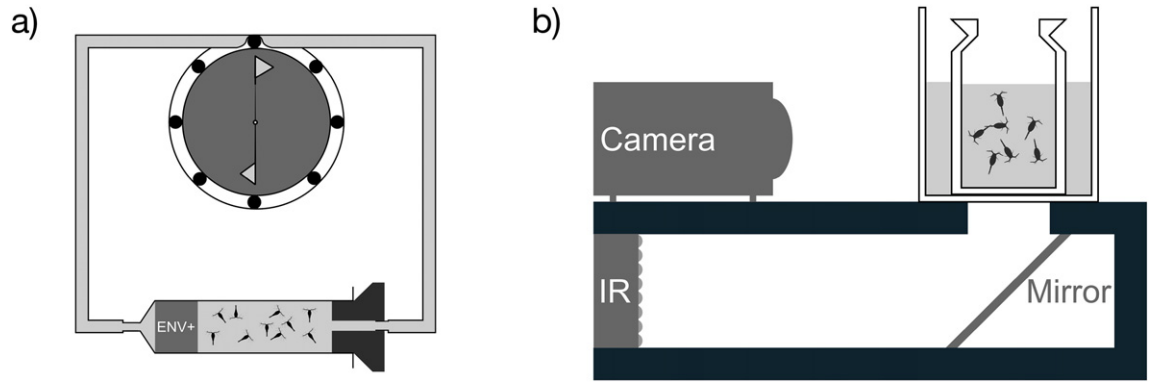

Fig. 1. a) Experimental setup of the extraction procedure. The copepods are placed inside the compartment of the SPE column (ENV+), and a circular current is provided by the peristaltic pump at low speeds. b) Illustration of the bioassay setup (dimensions not in scale) used to record the swimming behaviour of males in a scintillation vial containing female exudates. The vial is placed in a small water-filled transparent aquarium to compensate the optical distortion of the rounded vial. Infrared illumination is provided from below to give dark field illumination, and the copepods are filmed using an infrared sensitive camera (SONY DCR-TRV738E).

and sex, the scent of copepods thus also contains information about the individual reproductive state (juvenile, virgin, or mated) and probably the age of the partner.

Copepod exudates also trigger phytoplankton cells to initiate countermeasures to avoid predation. These responses include increases in toxin contents (Long et al., 2007; Bergkvist et al., 2008), adjustments of colony size (Bergkvist et al., 2012) and alterations of their swimming behaviour (Selander et al., 2011).

Despite the importance of pheromones and other infochemicals for copepods for all aspects of their life and regarding the related non-consumptive effects on lower trophic levels (Heuschele and Selander, 2014) not much is known about the sex specific differences of copepod exudates, except for one study on *Temora longicornis* (Selander et al., 2016). One reason for this is that the pheromone amounts necessary to elicit for example the trail following responses are very small and only represent a small fraction of their body weight (Bagøien and Kiørboe, 2005; Heuschele and Selander, 2014), which makes the detection against a complex sea water matrix difficult. While the main and abundant excretion products like ammonium and urea have been studied in copepods (reviewed in Gardner and Paffenhöfer, 1982; Regnault, 1987), those studies did not report on sex specific differences, with the exception of Butler et al. (1969) who found slightly higher rates of nitrogen and phosphorus exudates in females compared to males.

In this project we tested a) whether the ambush feeding cyclopoid *Oithona davisae* has a sex specific exudate signature similar to the one described in the cruising calanoid *Temora longicornis* (Selander et al., 2016) and b) if we can extract and capture the female sex pheromone that triggers the search behaviour in male copepods. To detect the differences in the chemical exudate signatures between treatments we used an untargeted metabolomics approach. We modified the experimental approach outlined in Selander et al. (2016) to improve signal to noise ratio in order to enable extractions from the substantially smaller copepods such as *Oithona davisae*. In addition we isolated copepods from laboratory cultures before they become sexually mature, as virgin females emit more pheromones (Heuschele and Kiørboe, 2012).

2. Materials and methods

2.1. Model species

As a model species we used *Oithona davisae* due to its clear sexual dimorphism in morphology and behaviour. It is a Pacific species and member of one of the most numerous copepod genera on the planet (Fransz and Gonzalez, 1995; Gallienne and Robins, 2001). It is a relatively small copepod, where males are significantly smaller than females. Virgin females trigger strong search reactions in males (Heuschele and Kiørboe, 2012), and compared to bigger species like *Temora longicornis* and *Calanus finmarchicus* it is feasible to incubate them separately in high numbers to ensure the virginity of the tested animals (Ceballos and Kiørboe, 2011). This may be important as some species do not emit pheromones when they have been mated (Heuschele and Kiørboe, 2012), which may compromise the efforts to sample pheromones (Selander et al., 2016).

We isolated individual copepodites of *O. davisae* from laboratory stock cultures that were kept at 20 °C at a salinity of 32 PSU. We separated individual C3–C5 copepodites into single wells of 24 well culture plates (Sarstedt 83.1836) filled with seawater supplemented with the prey species *Oxhyrris marina*. We replaced 1 ml of the food suspension every other day and determined copepods that had reached maturity. Virgin adult females and males from the well plates, as well as males from the stock culture were transferred into separate holding bottles, where they were kept until use in the extraction or bioassays.

2.2. Exudate extraction

The following protocol is based on the exudate extraction procedure described for *Temora longicornis* in Selander et al. (2016). We washed all glassware with methanol prior to usage to reduce the chemical background from other sources. To obtain a low and constant background of dissolved organic compounds in the incubation water we prepared a 2 l batch of purified sea water (pSW) that was subsequently used in all incubations. The water (32 psu) was suction filtered through a GF/F filter (Whatman) to remove particles and pumped (~7 ml min^{-1}) through two serial isolute ENV+ (Biotage) 200 mg solid phase extraction (SPE) columns, one on the inlet and one on the outlet of a piece of tubing connected to a peristaltic pump. The ENV+ resin consists of a polystyrene resin functionalized with phenolic groups. The SPE purification reduces the background of retainable dissolved organic compounds (DOC) and increases the signal to noise ratio in the samples from copepod incubations substantially (Selander et al., 2016). SPE columns were activated with 3 ml methanol followed by 5 ml distilled water prior to use.

Matured animals were transferred to a centrifuge tube with an 80 μm mesh bottom submerged in a glass beaker with pSW and left to empty their guts for a period of 30–45 min. Afterwards we dipped the tubes sequentially in two more beakers with pSW to eliminate carry-over of culture water, feed algae, or faecal pellets. The number of animals per replicate varied between 106 and 189 (see Table 1) depending on the availability of recently matured adults. Given that the sex-specific sorting of individual copepods was a bottleneck in our study, we had 3 separate extraction efforts. In each of these, we extracted exudates from two replicates for each treatment (see Table 1). One male replicate did for unknown reasons not show any exudate structures during the LCMS and was therefore excluded from the analysis.