Ecotoxicology and Environmental Safety 82 (2012) 80–84

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Electroantennogram measurement of the olfactory response of *Daphnia* spp. and its impairment by waterborne copper

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ARTICLE INFO

Article history:
Received 1 March 2012
Received in revised form
17 May 2012
Accepted 18 May 2012
Available online 19 June 2012

Keywords:
Daphnia
Electroantennogram
Olfaction
Metals
Chemical communication

ABSTRACT

In this study an electroantennogram (EAG) method was developed for use on live daphniids. The EAG response of *Daphnia magna* and *Daphnia pulex* to a variety of amino acids was measured. The strongest response measured was elicited by L-arginine and was shown to induce a concentration-dependent response indicating the response is olfactory in nature. Subsequent exposures of *D. magna* to a low, ecologically-relevant concentration of copper (7.5 μg/L) showed a disruption in EAG function. This study utilizes the development of an EAG method for measuring olfactory acuity of live daphniids and demonstrates that at ecologically-relevant concentrations, the olfactory dysfunction caused by copper can be detected. The EAG technique is a useful tool for investigating the olfactory response of daphniids to odourants at the cellular level and detecting the effects of toxicants on the olfactory acuity of daphniids.

1. Introduction

In aquatic animals olfaction is essential to mediate a variety of vital life activities such as finding food, choosing a mate, avoiding predators, and recognizing kin (Tierney et al., 2010). In daphniids, olfaction has been shown to be essential for locating food and avoiding predators (Roozen and Lürling, 2001; Hunter and Pyle, 2004). Till date, all research on the response of daphniids to odourants has been at the behavioral and whole-organism level. As the behavioral activity of daphniids can be difficult and time-consuming to measure, the development of a method to directly detect the neurophysiological response of a live, intact daphniid to odourants would represent an important advance.

The basis of olfaction in daphniids is not well understood; however, it has been extensively studied in other invertebrates (Sato et al., 2008; Chatterjee et al., 2009). Olfaction relies on the binding of an odourant molecule to receptors in olfactory sensory neurons (OSNs) (Park et al., 2002). The binding of an odourant molecule initiates a signal transduction cascade resulting in the opening of transmembrane channels and an influx of cations into the OSNs resulting in cellular depolarization (Park et al., 2002; Touhara, 2009). If there is a strong enough depolarization response, an action potential forms and propagates to the brain and induces a behavioral response (Park et al., 2002; Touhara, 2009). Similar receptors to those found in insects have been found in the genome of *D. pulex* and are believed to be involved in chemoreception (Peñalva-Arana et al., 2009). The influx of cations that results from the opening of channels in OSNs results in a change in the extracellular field potential surrounding the neurons, which can be measured using electrophysiolgical techniques (Touhara, 2009).

Previous olfaction studies using electrophysiological techniques have been conducted on fish using electro-olfactography (EOG) to measure the olfactory responses in various conditions (Baldwin et al., 2003; Sandahl et al., 2004, 2007; Green et al., 2010). Electroantennography (EAG) is the primary electrophysiological technique that is employed to measure olfactory acuity in invertebrates (Schneider and Seibt, 1969; Park et al., 2002). This technique was first described by Schneider (1957), and involved measuring the response of the antennae of the male silkworm moth to pheromones. This technique was later applied to other terrestrial and eventually aquatic invertebrates (Carr et al., 1987; Park et al., 2002).

Metals have been shown to have a profound effect on aquatic organisms. In particular, metals, such as copper, at ecologically-relevant concentrations can impair the response of *D. pulex* to the predator kairomone of *Chaoborus americanus* (Hunter and Pyle, 2004; Mirza and Pyle, 2009). Another daphniid species, *D. magna*, is commonly used in toxicity testing both in the environment and in the laboratory as an indicator species because they are very sensitive to metal contaminants (Gunatilaka et al., 2001). Consequently, understanding both the acute and chronic effects

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0147-6513/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2012.05.011

of metal exposure on trophically-important species, such as *Daphnia* spp., provides a robust foundation on which sound water quality guidelines for the protection of aquatic life can be developed.

The objectives of this research were to firstly, develop an EAG technique that could be used to measure the olfactory response in daphniids to standard chemosensory cues, such as amino acids. Secondly, we aimed to determine if contaminant-induced chemosensory dysfunction could be detected using the newly-developed technique. We used two daphniid species, *D. magna* and *D. pulex*, to demonstrate that the technique can be applied across species, and used a modified EAG technique originally developed for lepidopterans (Schneider, 1957). We then exposed both daphniid species to a short pulse of copper resulting in an impaired response to the same standard chemosensory cues.

2. Materials and methods

2.1. Culture methods

A *D. pulex* seed culture was obtained from Natural Resources Canada in Ottawa, Ontario, Canada and reared at 20 (± 2 °C) under a 16 h:8 h (light:dark) photoperiod in dechlorinated North Bay tap water (pH 8.72, hardness 72.5 ± 12.5 mg/L as $CaCO_3$, alkalinity 24.7 ± 0.6 mg/L as $CaCO_3$, copper 4.64 ± 1.86 μg/L). Water changes were conducted bi-weekly (50–100 percent), the *D. pulex* were fed *Chlorella emersonii* (1 mL/week, cell density: 1.26×10^{10} cells/mL), and given B_{12} and selenium supplements (1 mL/week of each).

Cultures of *D. magna* were obtained from Carolina Biological Supply Company (CBSC) in Burlington, North Carolina and the Lakehead University Aquatic Toxicology Research Center (ATRC) in Thunder Bay, Ontario. *Daphnia magna* were cultured in dechlorinated Thunder Bay tap water (pH 7.4, hardness 45.4 ± 0.1 mg/L as $CaCO_3$, alkalinity 50.4 ± 2.6 mg/L as $CaCO_3$, copper 2.4 ± 0.1 μg/L) at ambient room temperature (22.4 ± 0.3 °C) under a 16 h:8 h (light:dark) photoperiod. The lighting was from a full spectrum fluorescent light source. Water changes of 50–100 percent were conducted weekly. The *D. magna* were fed 3 mL per week of a *Nannochloropsis sp.* algae food source (cell density: 1.19×10^6 cells/mL) provided with the culture from CBSC.

2.2. Copper solutions

A 50 mg/L stock copper solution was prepared by dissolving $CuSO_4 \cdot 5\,H_2O$ (BDH Chemicals, VWR, Radnor, PA, USA) in ultrapure water obtained from a Milli-Q water source (EMD Millipore Corporation, Billerica, MA, USA). Three drops of trace metal grade nitric acid (Fisher Scientific, Ottawa, ON, Canada) were added to the stock solution (measured pH 3.74) to keep the metals dissolved. Copper exposure waters were prepared by diluting the stock solution in dechlorinated Thunder Bay tap water. The copper concentration (7.5 ± 0.9 μg/L) for each exposure was measured using the inductively coupled plasma atomic emission spectroscopy (ICP-AES). This concentration was selected because it can be expected to be found in a contaminated lake (Pyle et al., 2005) and it is near the safe copper concentration of 5 μg/L set by the Ontario Water Quality Objectives for the Protection of Aquatic Life (MOE (1999)). Quality control standards were measured using the same method at the beginning and end of the sample run to verify proper analytical performance and ensure accurate measurements.

2.3. Electroantennogram (EAG) measurements

Animals were clamped in a specially made *Daphnia* holding apparatus that was constructed using an acrylic sheet (22.5 cm × 15.5 cm) with a horizontal toggle clamp (Samona, Burnaby, BC, Canada). The daphniid was placed on a glass slide that was held in place on the base of the apparatus by parafilm. A stainless steel plate was placed on the abdomen of the subject and held in place with the toggle clamp. The stainless steel plate was attached to a grounding wire to ground the apparatus and the daphniid. The apparatus was angled (approximately 15°) to allow for irrigation water to efficiently flow over the daphniid. The parafilm and the stainless steel plate provided a channel for the dechlorinated water and subsequent olfactory stimulus to flow over the daphniid. A perfusion pinch valve apparatus (Warner Instruments Perfusion Pinch-Valve Apparatus, Hamden, CT, USA) was used to regulate the delivery of stimuli to the daphniid. With the water flowing between the plate and the parafilm, the daphniid was completely submerged during all experiments. The entire set up was enclosed in a Faraday cage and set on an anti-vibration table with stimulus and dechlorinated water entering from one side and waste exiting the other as shown in Fig. 1(i).

For the EAG recordings, glass electrodes with a pore diameter of 80–100 μm were made using a micropipette puller (MicroData Instruments, Inc., S. Plainfield, NJ, USA) and filled with a 4 percent gelatin and 0.9 percent NaCl solution. Gelatin-filled electrodes were stored in 3 M KCl and refrigerated at 4 °C until needed. Prior to experiments, electrodes were warmed to room temperature. Both the reference and the recording electrode were placed in Ag-AgCl half cell bridges (Type MEH810, World Precision Instruments, Inc., Sarasota, FL, USA) that were filled with 3 M KCl. Micromanipulators were used to obtain precise placement of the reference electrode on the eye and the recording electrode at the base of the antenna of the daphniid as shown in Fig. 1(ii). The difference between the signal at the recording and reference electrode was amplified 10 × by a DC headstage and differential amplifier (Model DP-311, Warner Instruments, Hamden, CT, USA). The signal was recorded using the PowerLab system (Model ML866, ADInstruments, Colorado Springs, CO, USA) and LabChart 7 software (v7.1, ADInstruments, Bella Vista, NSW, Australia). The data acquisition system was optimized and consistently yielded signals between 1.5 and 2.0 mV by the differential amplifier. The optimal settings for this measurement were controlled by the differential amplifier which had the high-pass filter (low frequency limit of amplifier) set at 0.1 Hz, the low-pass filter (high frequency limit of amplifier) set at 10,000 Hz, and the gain control set at 10. The LabChart software sampling rate was set at 20 samples per second.

2.4. Measurement of the Daphnia spp. EAG response to amino acids

Preliminary experiments were conducted to determine the responsiveness of both species to a variety of amino acids. Based on the EAG response of *D. pulex* to various concentrations of L-arginine, two concentrations (10^{-2} and 10^{-4} M) of this amino acid and 10^{-2} M L-alanine were used in the initial *D. magna* experiments. During the 1 hour assay on each individual daphniid ($n=5$), each

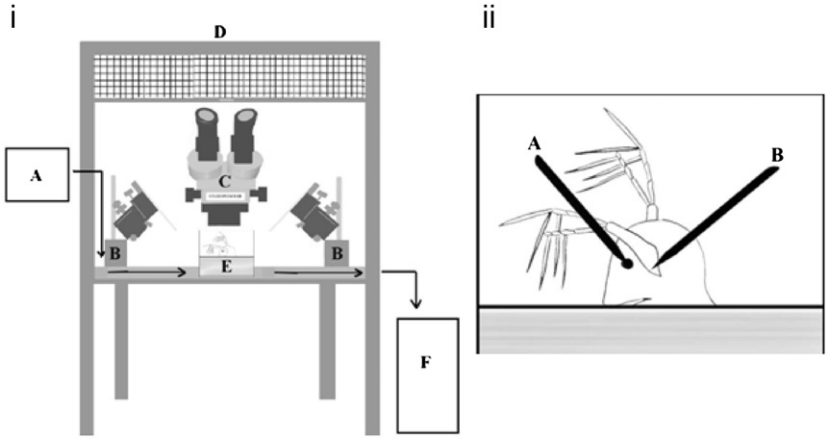

Fig. 1. (i) Schematic of the experimental design. Water and stimuli (A) enter through the left side of the Faraday cage (D), flow over the daphniid and into a waste collecting bin (F). Micromanipulators (B) and a microscope (C) were used to accurately place the electrodes on the daphniid which is clamped down by a stainless steel plate (E). (ii) Schematic of the placement of the electrodes on the daphniid during experiments. The reference electrode (A) is placed on the eye while the recording electrode (B) is placed at the base of the antenna.