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Research Paper

Learning on a chip: Towards the development of trainable biohybrid sensors by investigating cognitive processes in non-marine Ostracoda via a miniaturised analytical system

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Miniaturised analytical systems are showing growing interest in aquatic ecology, although this technology has not been exploited to study cognitive behaviours of organisms in aquatic micro-environments. Herein, a miniaturised testing arena was developed to investigate information processes and learning of *Heterocypris incongruens*, a freshwater ostracod relevant as bioindicator of environmental conditions. After dedicated training phases, a microchannel-based caging system enabled to tested if *H. incongruens* can associate a light stimulus to a food/stress source. Furthermore, the miniaturised system was used to test the ostracods ability in discriminating different coloured lights by choosing that previously associated with food or by avoiding the one previously associated with a stressor.

Trained ostracods significantly reacted to light stimuli compared to naïve individuals. When two different light colours were provided, trained ostracods selected the one associated with food, and avoided that associated with a stress source. The experiment in which ostracods were trained to associate light to food showed that *H. incongruens* not only exploits visual stimuli for decision making, but also for modulating its behaviour, swimming longer in presence of the right colour light than in presence of the different colour light, or no light. This can be an adapting behaviour balancing the energy use during foraging activities and limiting exposure to potential predators.

This study is the first to report such complex cognitive processes in ostracods, paving the way to new research directions for Lab-on-a-Chip systems, focused on behavioural ecology and cognition studies, as well as to the development of novel biohybrid sensors.

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1. Introduction

Miniaturised analytical systems are a recent technology milestone which have established themselves as one of the major instruments in many application fields, such as genomic and proteomic studies, analytical chemistry, diagnostic tests, environmental monitoring (Lafleur et al., 2016; Shanti et al., 2018; Temiz et al., 2015). With their relative cost effectiveness and miniaturised features, these devices allow the management of tests with little power and with portable devices, enabling accurate control of flowing liquids, reducing the amount of reagents/samples, decreasing reaction times, as well as standardised multiple experiments in parallel (Andar et al., 2019; Chiriacò et al., 2018; Lafleur et al., 2016; Manz et al., 1990; Sinha et al., 2019). The high design flexibility and the reduced time between the fabrication of these devices and the experiments have been also made possible by new emergent rapid-prototyping techniques including laser cutter machines, 3D printing, micromachining, laminated dry-film resists (DFRs) (Shahid et al., 2019; Temiz et al., 2015; Vasile et al., 1999).

Biomicrofluidics in aquatic ecology investigation represents a new and still little explored research field with a great potential in advancing these studies in controlled conditions, ensuring high repeatability and reproducibility (Campana & Wlodkowic, 2018; Mills et al., 2006).

Most of these investigations focused on aquatic ecotoxicology contexts, that benefited from a number of advantages offered by microfluidics, including dilution of toxicants, exposures based on micro-perfusion, and real-time analysis (Campana & Wlodkowic, 2018).

The largest amount of ecotoxicology studies relying on micro-engineered devices have been carried out on unicellular organisms (Brayner et al., 2011; Campana & Wlodkowic, 2018; Illing et al., 2016; Kim & Gu, 2003; Yoo et al., 2007). Furthermore, species of the phylum Nematoda have been widely exploited as model organisms in such studies (Clausell-Tormos et al., 2008; Jung et al., 2013, 2014; Kim et al., 2017; Zhang et al., 2014). Interestingly, a number of small vertebrates (Choudhury et al., 2012; Davies & Freeman, 1995; Lammer et al., 2009), and invertebrates (Zabihihesari et al., 2019), have also been used at embryonal stage.

Aquatic microarthropods are particularly suited to address ecological issues in freshwater and marine ecosystems (Pane et al., 2012; Verslycke et al., 2007; Won et al., 2017). Although their extensive use as animal models in ecotoxicology (Campana & Wlodkowic, 2018), a very small number of studies have focused on microfluidics to carry out experiments (Cartlidge et al., 2016, 2017; Huang et al., 2015a, b, 2016a, b).

Surprisingly, the great potential of miniaturised analytical platforms at micrometre/millimetre scale (Campana & Wlodkowic, 2018; Crane et al., 2010; Lafleur et al., 2016; Lee et al., 2012), has not yet been applied to study higher learning behaviours and cognition of these small organisms, in order to unveil overlooked features of their behavioural ecology.

Engineered systems can be useful for testing and modulating behavioural models in embodied microenvironments, allowing to investigate hypotheses about ecological mechanisms and interactions with environmental cues thanks to the replication of a biological phenomenon in an engineered device (Manfredi et al., 2013; Romano et al., 2019a; Romano & Stefanini, 2021).

Although the relatively small nervous systems of invertebrates, these organisms have shown elegant and complex mechanisms of learning, and a broad repertoire of behaviours (Perry et al., 2013; Shigeno et al., 2018). Learning abilities reported in invertebrates range from non-associative forms to associative learning (Davis & Heslop, 2004; McGuire, 1984). Other forms of learning includes higher order information processes enabling counting abilities, social learning, and more (Avarguès-Weber et al., 2018; Rapp et al., 2020). However, the ethology and cognitive abilities of aquatic micro-invertebrates still need to be clarified, also considering their importance in ecosystem functioning and as bioindicators. Early demonstrations of well-developed associative learning and persistence of learned patterns without reinforcement in invertebrates come from different crustacean lineages (Krasne, 1973; Reaka, 1980). Cephalops are well known for having a complex brain and storing of learned information (Mather & Kuba, 2013) and even consciousness (Mather, 2008).

Despite the accumulation of evidence on various types of learning across a large number of invertebrate phyla (Perry et al., 2013), the neural mechanisms of learning and memory are still poorly understood. For example, recent investigations seem to indicate that long-term memory may be transferred from trained to untrained animals by epigenetic modifications mediated by noncoding RNA, as in the case of marine mollusk *Aplysia* (Bédécarrats et al., 2018).

In this framework, a millimetre scale testing arena, including microfluidic channels, has been developed to investigate higher-order information processes and learning in the class Ostracoda (Crustacea), an arthropod group consisting of small to medium sized (0.3–7.0 mm) bivalved organisms. They are one of the most diverse and widespread of aquatic taxa, abounding in both marine and freshwater environments (Schön et al., 2003), whose neuroethology and behavioural ecology is rather undocumented (Mesquita-Joanes et al., 2012). Ostracods are of relevant interest in ecological and evolutionary studies, since their calcified carapaces in sea or lake sediments provide a real-time frame to their evolution (Martens et al., 2008), with a fossil record spanning at least 400–450 million years (Williams et al., 2008). Taxonomic identification of ostracods is notoriously rather difficult, because differences between species and genera are often based on small details of valve morphology and appendage chaetotaxy. In addition, the existence of cryptic species, i.e. individuals indistinguishable from each other from a morphological point of view, but genetically too different to be placed in the same species, have been confirmed in both recent marine and non-marine ostracods (Bode et al., 2010; Schön et al., 2012, 2017; Xu et al., 2019). There Q1 are two subclasses with living representatives: Myodocopa and Podocopa. The first subclass is exclusive to marine environments, with planktonic and benthic species, while Podocopa occur in marine, brackish and freshwater environments and are almost exclusively benthic.

Podocopa show a great variation in naupliar eye type, but the relationship between functions and different morphological designs is still largely unknown (Smith et al., 2015; Tanaka, 2005).

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Ostracods, thanks to their responses to particular parameters of the environment (Smith & Horne, 2002), also have an enormous importance as bioindicators and biosensors of environmental changes (Holmes & Chivas, 2002; Pieri et al., 2012), increasingly affected by unprecedented levels of anthropogenic impacts.

However, this class of aquatic arthropods are still unexploited by miniaturised analytical system technology. Herein, the ostracod *Heterocypris incongruens* (Ramdohr, 1808) (Podocopida: Cyprididae), a freshwater cosmopolitan organism exhibiting a wide feeding behaviour (Miličić et al., 2015), has been used as model organism. The aim of this study was to prove and quantify associative learning abilities in *H. incongruens* in rigorously controlled conditions enabled by interfacing our micro-engineered testing arena and this organism.

Information on ostracods learning ability can be exploited to produce a new paradigm of complex integrated miniaturised biosensors with "collaborative" trained bioindicators.

Furthermore, their potential sensing ability and learning skills, together with their well-documented large feeding spectrum (e.g. herbivory, detritivory, predation. omnivory, parasitism and even cannibalism) (Miličić et al., 2015; Rossi et al., 2011; Vannier et al., 1998) can be used to train *H. incongruens* individuals to prefer and process selected food sources. Thus, trained strains of ostracods would play key roles in detecting, as well as in decomposing particular organic materials in eutrophic areas.

2. Materials and methods

2.1. Ethics statement

This research complied with the guidelines provided by ASAB/ABS (2015) concerning the treatment of animals in behavioural research and teaching, the Italian law (D.M. 116192), and the European Union regulations (European Commission, 2007). No authorisations are required in Italy to conduct behavioural observations on *H. incongruens*.

2.2. Heterocypris incongruens *rearing and general information*

Wild individuals of *H. incongruens* were collected in late summer in a permanent pond in Pontedera (Pisa, Tuscany, Italy). Adult individuals were used to determine the specific allocation of the collected material. The species identification was evaluated by checking both soft parts and valves, based on Meisch (2000). *H. incongruens* were reared for several months in different tanks (300 × 300 × 200 mm) containing aged tap water, in laboratory conditions (20–22 °C, 16 : 8 L: D photoperiod, with light intensity in close proximity of the tank of approximately 1000 lux, estimated over the 300–1100 nm waveband). Ostracods were fed with a diet composed of dried "*Spirulina*", a filamentous cyanobacterium commercialised as food for fish, which also provides a convenient substrate for valve moulting and egg-laying.

The miniaturised testing arena and its parts were carefully washed after each replicate, for about 30 s, with warm water (e.g. 35–40 °C), cleansed with water plus mild soap for approximately 5 min, rinsed with hot water for about 60 s, then rinsed with tap water at room temperature, and finally refilled with dechlorinated tap water at 20 ± 2 °C (Benelli et al., 2015; Romano et al. 2019b).

2.3. Miniaturised testing arena

The miniaturised testing arena was designed in SolidWorks (Dassault Systemes, Vélizy Villacoublay, France) and then fabricated by rapid prototyping in a bio-compatible resin (VisiJet® M3 Crystal, 3D Systems), to carry out behavioural experiments based on a Lab-on-Chip (LOC) approach.

It consists of a lower component and an upper component (Fig. 1a). The lower component has a diameter of 70 mm, height 15 mm, and presents 3 through holes (diameter 6 mm), each of which can house a Light Emitting Diode (LED). Previous studies revealed that marine podocopid ostracods possess eyes that are adapted to different light conditions, and light-gathering ability is considerably different between species and is related to morphological features of the optical systems (Tanaka, 2006). Since no specific information was available regarding the light-gathering ability of *H. incongruens*, for the experiments we chose two wavelengths in the visible spectrum that can be linked to the visual ecology of this species, and that are perceived by many arthropod colour vision systems (Briscoe & Chittka, 2001; Oakley & Huber, 2004; Osorio & Bacon, 1994). A yellow LED (wavelength 587–595 nm) and a green LED (wavelength 520–525 nm) were used. Light intensity of both LEDs was 1 mcd. The position of the LEDs could be shifted among different exploration chambers. The upper component represents the real testing arena. It has a diameter of 70 mm, height 10 mm, and include a releasing chamber (diameter 20 mm; height 10 mm) and three exploration chambers (diameter 10 mm; height 10 mm). These chambers created small environments to analyse *H. incongruens* behaviour but did not restrict the swimming activity of ostracods avoiding potential bias due to spatial constraints. Each exploration chamber is connected to the releasing chamber through an aisle (5 × 5 mm; height 10 mm). The floor of the testing arena is represented by a transparent plexiglass disk firmly connected to the base of the upper component. During the experiments, the upper component was placed on the lower component in order to have the three exploration chambers perfectly centred with the three through holes of the lower component.

H. incongruens individuals, thanks to their long natatory setae on the antenna allowing short swims away from the sediment, spend most of time in exploring rapidly the environment (Miličić et al., 2015), as also confirmed by our personal observations.

To effectively cage free swimming ostracods that moved from the releasing chamber to an exploration chamber, a removable membranous partition was located in each aisle.

The membranous partition consists in a membrane holder (VisiJet M3 Crystal), and a transparent membrane (thickness 1000 μm) in polydimethylsiloxane (PDMS) presenting 3 microchannels. The PDMS used (Sylgard 184, Dow Corning), is commonly employed as substrate for cell cultures, thus it is highly bio-compatible (Cafarelli et al., 2017), and reasonably non-toxic toward Ostracoda and other organisms. The

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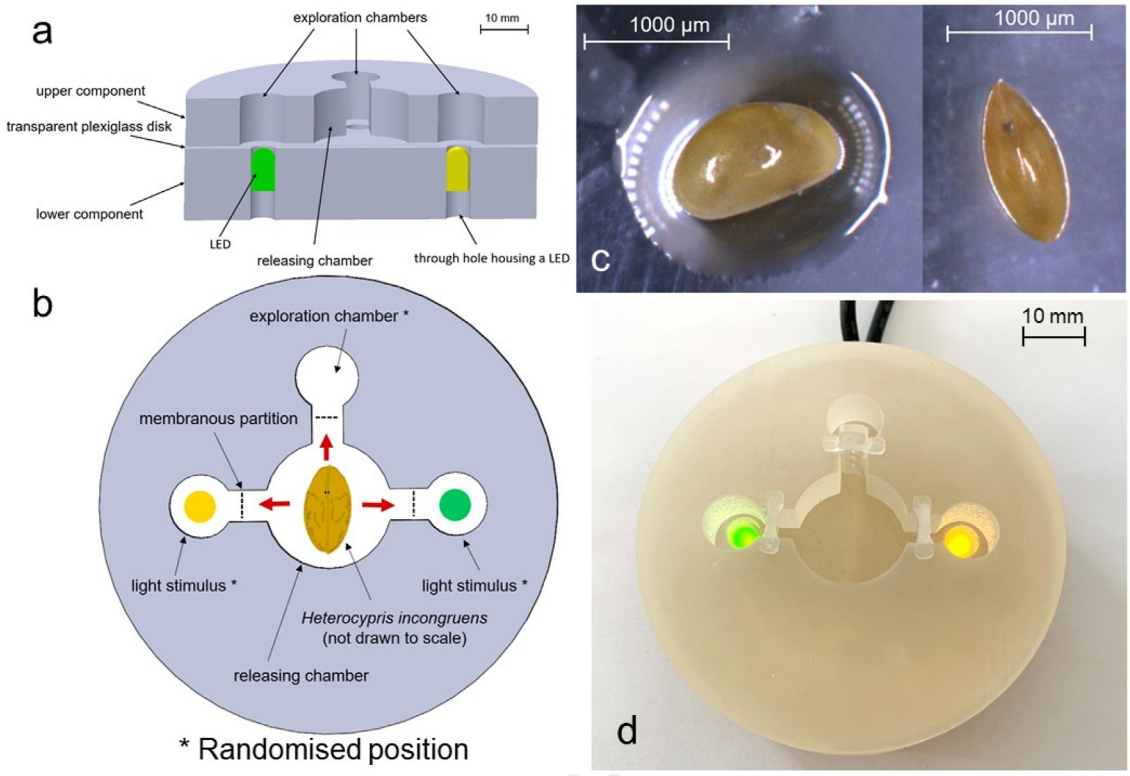

Fig. 1 – Cross-section architecture of the miniaturised testing arena (a). Setup of the LOC-based behavioural experiments (b). Lateral view (left) and dorsal view (right) of *Heterocypris incongruens* (c). Miniaturised testing arena (d).

transparent PDMS membranous partition do not affect the light intensity. The conical frustum microgeometry of such microchannels ($r1 = 500$ µm; $r2 = 250$ µm), allows a one-way passage of ostracods from the releasing chamber to an exploration chamber, enabling *H. incongruens* choice identification. The fabrication process of the membranous partition is depicted in Fig. 2. LEDs activation in the lower component was enabled by an off-arena microcontroller (Arduino, Mega 2560).

The behaviour of *H. incongruens* in the miniaturised testing arena was observed under a 3D visual inspection microscope (magnification 10x) (Mantis Elite, Vision Engineering, England).

2.4. *Training phase for the LOC-based behavioural experiments 1*

Adult *H. incongruens* were kept individually in a Petri dish (diameter 40 mm) filled with water from their aquarium, in the same laboratory conditions described above. At each feeding event (e.g. delivering of *Spirulina*-based food three time a day), a light stimulus was also presented (e.g. yellow or green LEDs positioned below the Petri dish floor). Each individual was trained with just one colour. The training phase, for the in LOC-based behavioural experiments 1, lasted three days, where the light stimulus stayed on for one hour from the introduction of food and then was turned off together with the removal of leftover food. A portion of *H. incongruens* were feed without coloured light stimuli (naïve) as control.

2.5. *LOC-based behavioural experiment 1*

Here, if *H. incongruens* can associate a light stimulus to a food source was investigated. Furthermore, the ability of these animals to discriminate between two lights of different colours by choosing the one previously associated with a food source.

Adult ostracods were transferred in the releasing chamber of the miniaturised testing arena by using a micropipette. Each animal was tested individually and only once to avoid effects due to other conspecifics presence that would affect *H. incongruens* choices, and masking learning processes. After 60 minutes of acclimation (Miličić et al., 2015), light cues were activated and the test started (Fig. 1b, c, d).

Trained and naïve *H. incongruens* subjects were exposed to the following treatments: i) light stimulus previously associated with food (e.g. correct colour light), ii) light stimulus different from the one previously associated with food (e.g. different colour light), iii) both right and different colour lights exposed at the same time (e.g. both colour lights).

The treatments i) and ii) consisted in a two-choice test (e.g. access to the median exploration chamber was avoided by placing a partition in its aisle). The treatment iii) consisted in a three-choice test.

The position of light stimuli was shifted among different exploration chambers after each replicate, to avoid directional bias.

The choice of individuals was evaluated and recorded by observing in which exploration chamber ostracods were

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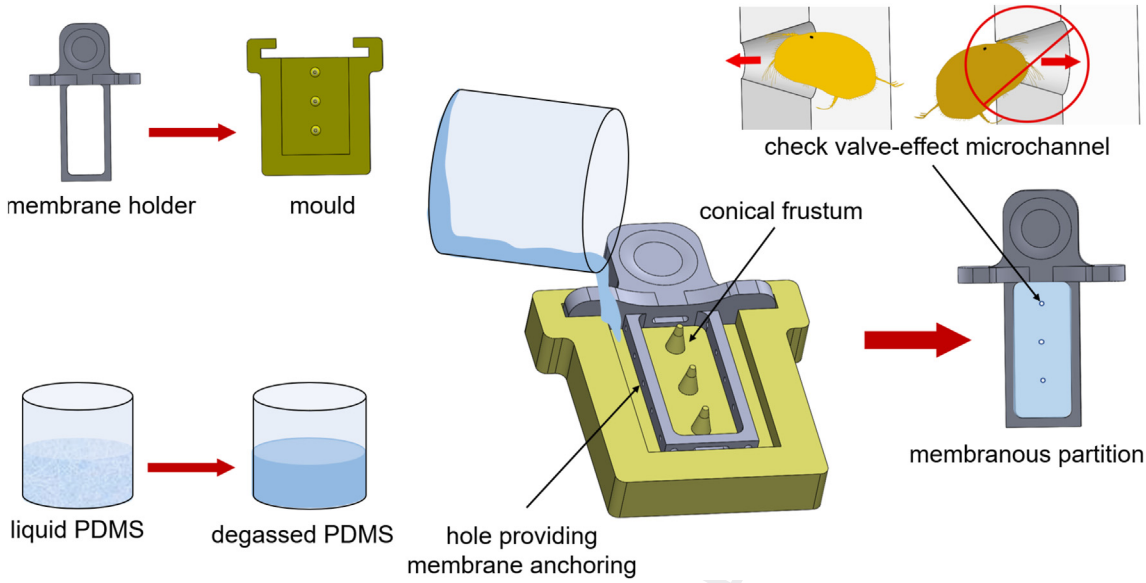

Fig. 2 – Fabrication process of the membranous partition. Degassed PDMS is poured into a mould presenting conical frustums and including the membrane holder. The membrane holder is removed from the mould once the PDMS membrane is cured anchoring to it. The conical frustums of the mould enable the formation of microchannels in the membrane with a check valve-effect microgeometry to cage ostracods in the selected exploration chamber (97% effective on 100 individuals tested in preliminary tests).

caged. The time needed to make a choice (duration from the starting of the experiment and the entry in an exploration chamber) was also measured.

Once an ostracod entered an exploration chamber it was observed for 10 minutes and the duration of the swimming activity, as well as of the resting behaviour were recorded.

Before each test, individuals were starved for 3 h (Milićić et al., 2015).

For each treatment, 100 trained ostracods and 100 naïve ostracods were tested. Overall, 600 *H. incongruens* were analysed.

2.6. *Training phase for the LOC-based behavioural experiment 2*

H. incongruens specimens, individually kept in a Petri dish (diameter 40 mm) filled with water from their aquarium (conductivity c. 490 μS cm^{-1} at 20 °C), were transferred for one hour, three times a day, to another identical Petri dish containing water with NaCl to have a salinity level of 9‰ (conductivity c. 13,900 μS cm^{-1} at 20 °C), and that also presented different light stimuli (e.g. yellow or green LEDs positioned below the Petri dish floor). This salinity level can potentially elicit a repulsive associative response with light stimuli. The selected salinity can be considered an unfavourable but not harmful abiotic condition for *H. incongruens*, whose natural populations can be occasionally found at comparable or even higher salinity levels, although it largely prefers low salinity waters (Meisch, 2000; Pieri et al., 2020; Ruiz et al., 2013). Each individual was trained with just one colour. As control, a portion of *H. incongruens* were exposed to high salinity level without coloured light stimuli (naïve). The training phase, for the in LOC-based behavioural experiments 2, lasted three days.

2.7. *LOC-based behavioural experiment 2*

In this experiment, we investigated if *H. incongruens* can associate a light stimulus to a stress source. The ability of *H. incongruens* individuals in discriminating two lights different in colour, by avoiding the one previously associated with a stress source, was also evaluated.

The procedure of the behavioural experiment 2 was similar to that of the behavioural experiment 1. Here, treatments that were presented to trained and naïve *H. incongruens* subjects included: iv) light stimulus previously associated with salt water (e.g. right colour light), v) light stimulus different from the one previously associated with high salinity water (e.g. different colour light), vi) both right and different colour lights exposed at the same time (e.g. both colour lights). The position of light stimuli was shifted among different exploration chambers after each replicate, to avoid directional bias. The choice of individuals was evaluated and recorded by observing in which exploration chamber ostracods were caged. Overall, 600 individuals were analysed.

2.8. *Statistical analysis*

For treatments i), ii), iv), and v) the difference in the number of ostracods caged in different exploration chambers was analysed with a χ^2 test with Yates' correction ($P < 0.05$).

The impact of the treatments iii) and vi) on the choice of trained and naïve *H. incongruens* caged in different exploration chambers was analysed using the generalised linear model (glm) with a binomial error structure described in Romano et al. (2018). Significant differences among values were evaluated by using a probability level of $P < 0.05$.

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Data concerning the impact of treatments i), ii), and iii) on the time needed to make a choice, the swimming activity, and the resting behaviour were analysed with non-parametric statistics based on the Wilcoxon test ($P = 0.05$), as these data were not normally distributed (Shapiro-Wilk test, goodness of fit $P < 0.05$). R software v3.6.1 (R Development Core Team, 2019) was used to analyse all data.

3. Results

3.1. LOC-based behavioural - experiments 1

Herein, *H. incongruens* successfully proved to be able to associate a light stimulus to a food source, as well as in discriminating two lights different in colour by choosing the one associated with a food source, as learned in previous training phases.

The number of trained ostracods selecting the exploration chamber with the light colour previously associated to a food source (correct colour), was significantly higher than the number of trained ostracods that selected the exploration chamber with no light (correct colour versus no light: 87 versus 13; $\chi^2_1 = 54.77$; $P < 0.00001$) (Fig. 3a).

The number of naïve ostracods selecting the exploration chamber with the right light colour was not significantly different than the number of naïve ostracods that selected the exploration chamber with no light (right colour versus no light: 59 versus 41; $\chi^2_1 = 3.25$; $P < 0.071423$) (Fig. 3a).

The number of trained ostracods selecting the exploration chamber with a light colour differently from the one previously associated to a food source (i.e. different colour), was significantly higher than the number of trained ostracods that selected the exploration chamber with no light (different colour versus no light: 76 versus 24; $\chi^2_1 = 27.05$; $P < 0.00001$) (Fig. 3a).

The number of naïve ostracods selecting the exploration chamber with the different light colour was not significantly diverse than the number of naïve ostracods that selected the exploration chamber with no light (different colour versus no light: 56 versus 44; $\chi^2_1 = 1.45$; $P < 0.228528$) (Fig. 3a).

In the treatment iii), trained individuals' choice was significantly affected by different stimuli presented in the miniaturised testing arena ($\chi^2 = 123.90$, *d.f.* $= 2$, $P < 0.0001$) (Fig. 3b).

Trained ostracods were significantly more attracted by the correct colour light than the different colour light ($\chi^2_1 = 46.02$, $P = 1.169831e^{-11}$) and preferentially attracted by the correct colour light than no light ($\chi^2_1 = 119.08$, $P = 1.002759e^{-27}$); in addition, they were significantly more attracted by the different colour light than the no light ($\chi^2_1 = 22.56$, $P = 2.026313e^{-6}$) (Fig. 3b).

In the treatment iii) naïve individuals' choice was not affected by stimuli to which they were exposed in the miniaturised testing arena ($\chi^2 = 1.82$, *d.f.* $= 2$, $P = 0.4006$) (Fig. 3b).

The time needed to make a choice was significantly affected by previous experience and by stimuli provided by different treatments ($\chi^2 = 388.29$, *d.f.* $= 5$, $P < 0.0001$). The duration from the starting of the experiment and the entry in an exploration chamber was significantly shorter in trained ostracods than naïve ostracods for treatment *i* ($Z = -11.14$; $P < 0.0001$), treatment ii ($Z = -10.07$; $P < 0.0001$), and treatment iii ($Z = -11.94$; $P < 0.0001$). Trained ostracods exposed to treatment *i* exhibited a faster choice than trained ostracods in treatment ii ($Z = -7.12$; $P < 0.0001$), and treatment iii ($Z = -3.48$; $P = 0.0005$). Trained ostracods exposed to treatment ii exhibited a slower choice than trained ostracods in treatment iii ($Z = -5.98$; $P < 0.0001$) (Fig. 4a).

Previous experience and different treatments significantly affected the swimming activity duration of *H. incongruens* ($\chi^2 = 237.11$, *d.f.* $= 5$, $P < 0.0001$). Swimming duration lasted longer in trained ostracods than naïve ostracods in response to treatment *i* ($Z = 10.47$; $P < 0.0001$), treatment ii ($Z = 6.44$; $P < 0.0001$), and treatment iii ($Z = 8.07$; $P < 0.0001$). Trained ostracods exposed to treatment *i* exhibited a longer duration of swimming activity compared to trained ostracods in treatment ii ($Z = -6$; $P < 0.0001$), and treatment iii ($Z = 2.81$; $P = 0.0049$). Trained ostracods exposed to treatment *ii* showed a shorter duration of swimming activity than trained ostracods in treatment iii ($Z = -3.7$; $P = 0.0002$) (Fig. 4b).

The resting behaviour of *H. incongruens* was significantly affected by previous experience and different treatments ($\chi^2 = 237.12$, *d.f.* $= 5$, $P < 0.0001$). Resting behaviour lasted longer in naïve *H. incongruens* than in trained *H. incongruens* exposed to stimuli of treatment *i* ($Z = -10.47$; $P < 0.0001$), treatment ii ($Z = -6.44$; $P < 0.0001$), and treatment iii ($Z = -8.07$; $P < 0.0001$). Trained ostracods exposed to treatment *i* showed a shorter resting behaviour than trained ostracods exposed to treatment ii ($Z = 6$; $P < 0.0001$), and treatment iii ($Z = -2.81$; $P < 0.0050$). Trained ostracods exposed to treatment *ii* exhibited a longer resting time than trained ostracods in treatment iii ($Z = 3.7$; $P = 0.0002$) (Fig. 4c).

3.2. LOC-based behavioural - experiments 2

The LOC-based behavioural experiments 2 showed that *H. incongruens* are also able to associate a light stimulus to a stress source. Furthermore, *H. incongruens* discriminated two lights different in colour by avoiding the one that during the training phase was associated with a stress source.

The number of trained ostracods accessing the exploration chamber with the light colour previously associated to a stress source (correct colour), was significantly lower than the number of trained ostracods that selected the exploration chamber with no light (correct colour versus no light: 8 versus 92; $\chi^2_1 = 70.57$; $P < 0.00001$) (Fig. 5a).

The number of naïve ostracods selecting the exploration chamber with the correct light colour did not vary significantly from the number of naïve ostracods that selected the exploration chamber with no light (correct colour versus no light: 57 versus 43; $\chi^2_1 = 1.97$; $P < 0.160448$) (Fig. 5a).

The number of trained ostracods accessing to the exploration chamber with a light colour different from the one previously associated to a stress source (different colour) was significantly lower than the number of trained ostracods accessing to the exploration chamber with no light (different colour versus no light: 22 versus 78; $\chi^2_1 = 31.37$; $P < 0.00001$) (Fig. 5a).

The number of naïve ostracods selecting the exploration chamber with the different light colour, was not significantly

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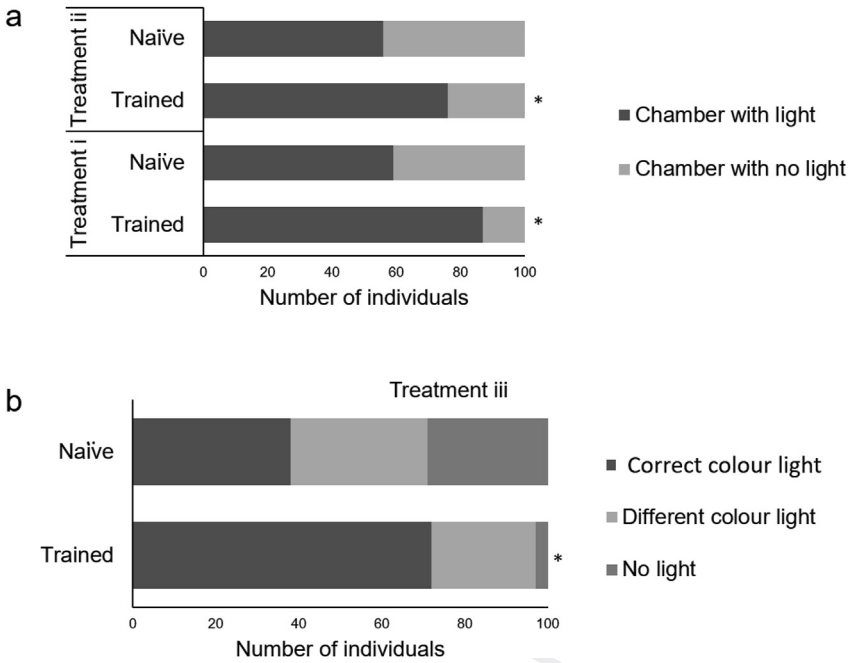

Fig. 3 – Number of naïve and trained *H. incongruens* individuals, exposed to treatment i) and ii), that were caged in the exploration chamber of the miniaturised testing arena with the light stimulus, and with no light stimulus (a). Number of naïve and trained *H. incongruens* individuals, exposed to treatment iii), that were caged in the exploration chamber of the miniaturised testing arena with the right colour light stimulus, the different colour light stimulus, and with no light stimulus (b). Asterisks (*) indicate statistically significant preferences.

different than the number of naïve ostracods that selected the exploration chamber with no light (different colour versus no light: 52 versus 48; $\chi^2_1 = 0.17$; $P < 0.680112$) (Fig. 5a).

During the treatment vi) the choice of trained *H. incongruens* was importantly influenced by different stimuli presented in the miniaturised testing arena ($\chi^2 = 166.85$, $d.f. = 2$, $P < 0.0001$) (Fig. 5b). Trained ostracods avoided more the correct colour light than no light ($\chi^2_1 = 125.22$, $P = 4.548599e^{-29}$), as well as they avoided more the different colour light than no light ($\chi^2_1 = 116.68$, $P = 3.369551e^{-27}$). There were no significant differences between the number of *H. incongruens* attracted by the correct colour light and the different colour light ($\chi^2_1 = 0.24$, $P = 0.620860$) (Fig. 5b).

In the treatment *vi* the choice of naïve individuals was not affected by the stimuli to which they were exposed in the miniaturised testing arena ($\chi^2 = 1.1$, $d.f. = 2$, $P = 0.5744$) (Fig. 5b).

4. Discussion

The idea that invertebrates are "mindless machines" has long since rejected (Zylinski, 2015). Nevertheless, observatory learning in invertebrates is difficult to assess under natural conditions (Menzel et al., 2007). Using engineered testing arenas can help in overcoming these problems. In particular, miniaturised analytical platforms are gaining a momentum in ecological studies focused on aquatic micro-environments (Campana & Wlodkowic, 2018; Cartlidge et al., 2017; Illing et al., 2016). The great potential of these devices can enable investigations on the behavioural ecology and learning mechanisms of microarthropods of relevant importance as bioindicators and biosensors. However, this aspect has been overlooked so far.

As in vertebrates, invertebrates can modify their behaviour by learning processes (Avarguès-Weber et al., 2018; Nargeot & Bédécarrats, 2017). Learning abilities reported in invertebrates include both non-associative and associative forms (Davis & Heslop, 2004; McGuire, 1984). Animals use associative learning to establish predictive relationships between events including sensory stimuli and motor actions (Bower & Winzenz, 1970), and can be distinguished in classical or respondent conditioning, and operant or instrumental conditioning (Nargeot & Bédécarrats, 2017). Invertebrates have been found to be elective model organisms to analyse neuroethological basis of learning, as they have relatively simple behaviours that can be modulated by different associative learning procedures, similar to those employed by vertebrates (Hawkins & Byrne, 2015; Perry et al., 2013). Furthermore, the neuronal architecture producing these behaviours include a relatively small numbers of neurons that are easier to identify and to analyse at cellular level (Moroz, 2011).

Herein, we presented a LOC-based testing arena with microfluidic channels, to investigate higher-order information processes and learning in the ostracod *H. incongruens*. Previous studies investigated phototaxis display in other freshwater crustacean by using microfluidic devices (Cartlidge et al., 2016). In this study a miniaturised testing platform was first used to shade light on unexplored learning processes of aquatic micro-arthropods.

The results of this research unveiled the ability of *H. incongruens* in associating a light stimulus with a food source. Freshwater ostracods are active foragers (Roca et al., 1993), but

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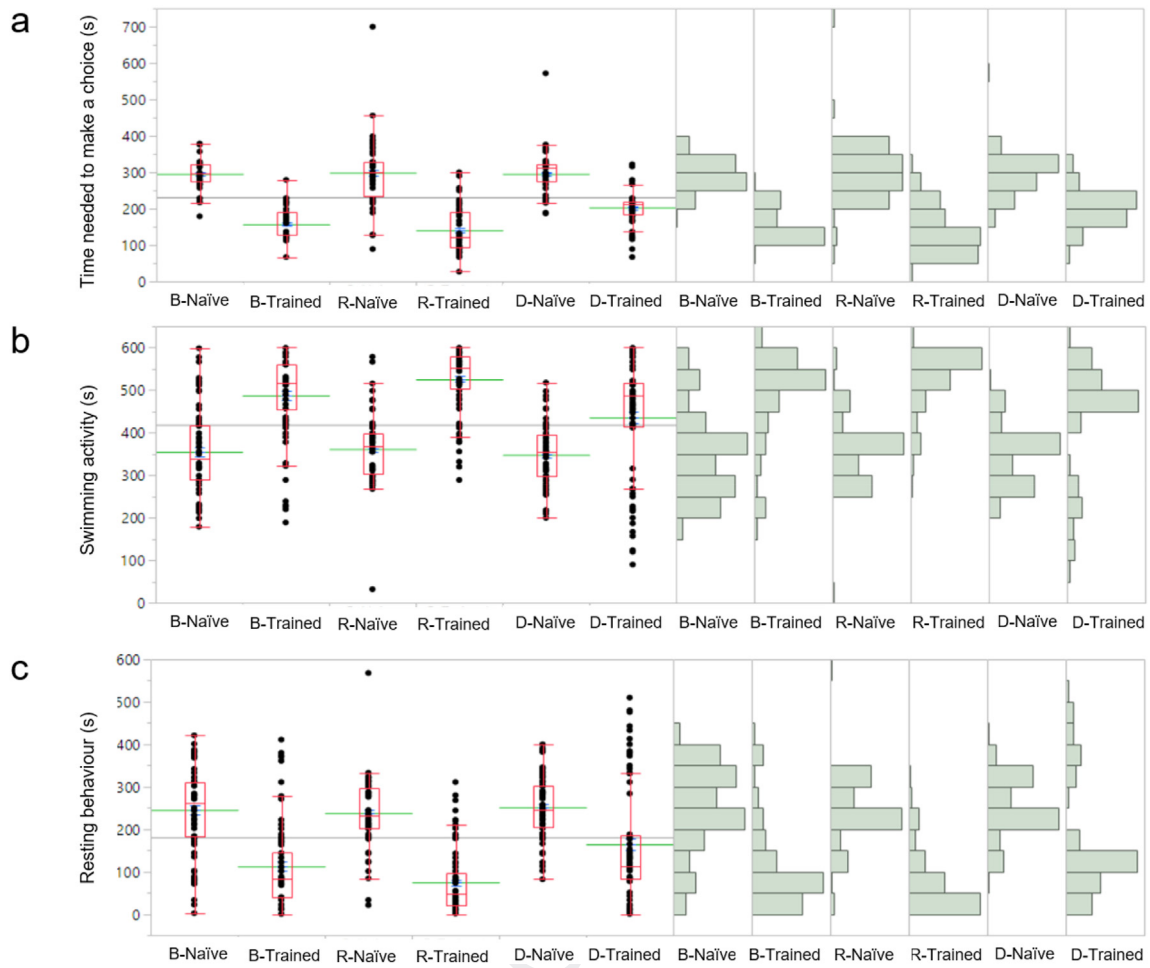

Fig. 4 – Time needed to make a choice (a), duration of the swimming activity (b), and duration of the resting behaviour (c) in *H. incongruens* post-exposure to different treatments. B-Naïve: naïve individuals exposed to both colour lights; B-Trained: trained individuals exposed to both colour lights; R-Naïve: naïve individuals exposed to the correct colour light; R-Trained: trained individuals exposed to the correct colour light; D-Naïve: naïve individuals exposed to the different colour light; D-Trained: trained individuals exposed to the different colour light. The median (red line) and their lower and upper quartiles and outliers, as well as green lines (mean value) and blue T-bars (standard error value) are reported in the box plots. Data distributions are shown by histograms on the right of each box plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

this high degree of locomotion activity is energy costly (Ydenberg & Dill, 1986), as well as can overexpose them to potential predators (Uiblein et al. 1992, 1996). *H. incongruens* individuals demonstrated how they can quickly learn from a previous training phase and adapt their decision-making behaviour to select microhabitats allowing them to balance food searching effort and predation risk (Kohler & McPeek, 1989; Roca et al., 1993).

H. incongruens was also able to avoid a light stimulus associated with a stress source, represented by an increased salinity level. Salinity is a major factor affecting the structure of aquatic communities in freshwater ecosystems. Variations of abiotic factors, including salinity, has been reported to significantly regulate the fitness of *H. incongruens*, and of ostracods in general (Bieszke et al., 2020; De Deckker, 1981; Laut et al., 2016). The behavioural responses observed with the proposed biohybrid paradigm remarkably provide the evidence that *H. incongruens* learning ability is an elective aspect bio-indicating the water quality (Kim et al., 2015; Lawrence et al., 2002; Ruiz et al., 2013).

Notably, the caging system provided by the miniaturised testing arena revealed how this ostracod species can also discriminate lights different in colour by identifying those previously associated with a food source, as well as those associated with a stress source.

Some ostracod species in the subclass Myodocopa evolved functional iridescence and bioluminescence (Oakley, 2005; Parker, 1995), as well as lateral compound eyes to better detect conspecifics in deep water (Parker, 1995). On the contrary, visual signalling through bioluminescence as mate recognition system has never been documented in non-marine ostracods. A probable use of vision for mate recognition is present in species of *Notodromas*, a genus of hyponeustonic non-marine ostracods, therefore dwelling in an illuminated environment, while the vast majority of non-marine ostracods are benthic. Other important features in *Notodromas*

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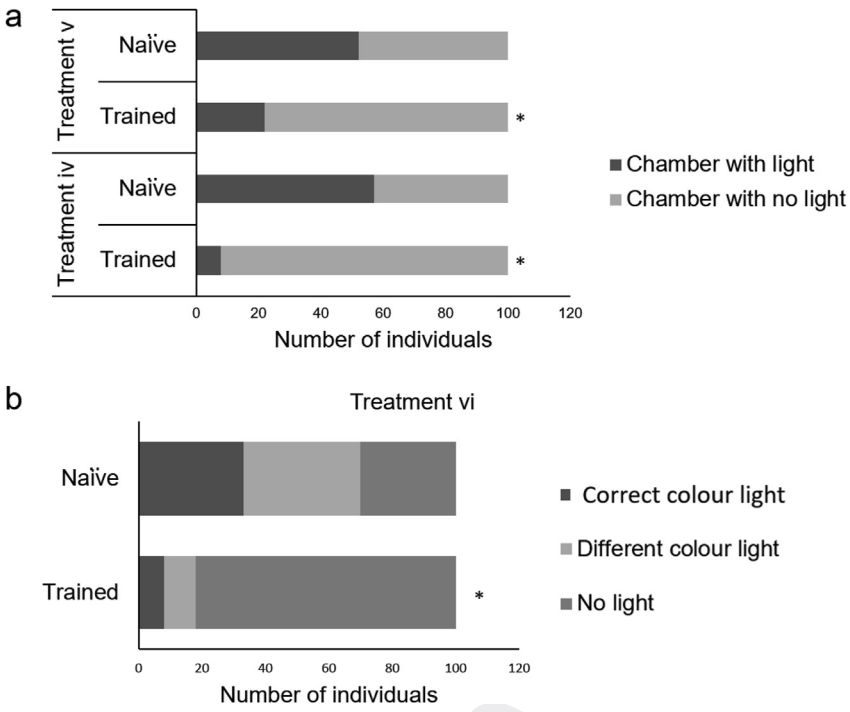

Fig. 5 – Number of naïve and trained *H. incongruens* individuals, exposed to treatment iv) and v), that were caged in the exploration chamber of the miniaturised testing arena with the light stimulus, and with no light stimulus (a). Number of naïve and trained *H. incongruens* individuals, exposed to treatment vi), that were caged in the exploration chamber of the miniaturised testing arena with the right colour light stimulus, the different colour light stimulus, and with no light stimulus (b). Asterisks (*) indicate statistically significant preferences.

species are the presence of a naupliar eye ramified in three ocelli, and of biconvex lenses, more developed in males, located on the valves in positions corresponding to the lateral ocelli. Also, females are strongly pigmented (Home et al., 1998). In addition, our results indicate the ability of the podocopid *H. incongruens* in exploiting light sources through its translucent valves to increase its fitness.

The higher phylogeny of ostracods is still unconfirmed. Several authors have suggested that ostracods are a monophyletic group (McKenzie, 1972; Oakley et al., 2013; Parker, 1995) and that the myodocopids branched off the podocopid tree in the Ordovician (McKenzie, 1972; Parker, 1995; Siveter & Vannier, 1990), whereas others consider Myodocopa and Podocopoda not closely related and consequently ostracods as a polyphyletic group (Wakayama, 2007; Horne et al., 2005). Regardless of these alternative views, this study demonstrates the ability to process and exploit light stimuli also in podocopids inhabiting shallow waters. Phototactic responses, positive or negative, were already reported in marine podocopid ostracods (Tanaka, 2006). Furthermore, the influence of illumination on the spatial orientation in *H. incongruens* and *Notodromas monacha* (O.F.Müller 1776) was evaluated under microgravity conditions (Fischer & Laforsch, 2018). Laboratory experiments showed the effect of photoperiod on life-history traits of *H. incongruens* (Rossi & Menozzi, 1993). Findings reported here further confirm how Ostracoda are an important model for studying the evolution of vision and light-related features (Oakley, 2005).

In addition, *H. incongruens* not only can exploit visual stimuli to make a decision but can also use them to modulate its behaviours, as observed in the experiment 1. Feeding behaviour crucially affects processes related to development, morphology, physiology, and ecological features of a species (Miličić et al., 2015). Trained *H. incongruens* were more active in presence of the right colour light than in presence of the different colour light, or no light, showing a probable attempt in dosing energy for foraging activity and limiting exposure to potential predators when swimming in search of suitable food (Kohler & McPeek, 1989; Roca et al., 1993; Uiblein et al., 1992, 1996; Ydenberg & Dill, 1986).

Ostracods show clear microhabitat preferences which are influenced by habitat structure and food supply (Mbahinzireki et al., 1991; Wilkinson et al., 2007). Freshwater ostracods are usually thought to explore the surrounding habitat by receptors (e.g., modified setae) sensitive to mechanical and chemical stimuli (Smith & Matzke-Karasz, 2008). Even vision may have a role that has been underestimated so far.

Our findings represent the first evidence of such complex cognitive processes in *H. incongruens*, and in ostracods in general. Our results can pave the way to a new research direction for miniaturised analytical systems focused on behavioural ecology and cognition of aquatic micro-invertebrates.

Furthermore, our results show how learning processes exhibited by ostracods, along with their important role as bioindicators, can enable the use of these animals, interfaced with miniaturised devices, as trainable organism-based

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sensors for *in vitro* biomonitoring tasks. Namely, several studies confirm the potential of *H. incongruens* as test-organism in ecotoxicological essays (Belgis et al., 2003; Chial & Persoone, 2002; Gosset et al., 2016; Muna et al., 2019).

5. Conclusions

This study demonstrates the novel and key role of miniaturised engineered devices to investigate learning processes of organisms in aquatic micro-habitats.

Herein we unveiled the ability of *H. incongruens* in associating light stimuli with food and stress sources. *H. incongruens* individuals were also able to discriminate two lights different in colour. This research shows how these microarthropods can learn from previous experience and adapt their decision-making behaviour to identify selected microhabitats. These findings represent the first evidence of such complex cognitive processes in Ostracoda. Furthermore, freshwater ostracods are usually thought to explore the surrounding environment by using mechanical and chemical receptors. Further research is needed to understand the visual ecology of these species.

Overall, our results show how aquatic microarthropods' learning processes, along with their important role as bio-indicators, can lay the foundations for new research directions towards the development of trainable organism-based sensors for biohybrid biomonitoring tasks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES

Andar, A., Hasan, M. S., Srinivasan, V., Al-Adhami, M., Gutierrez, E., Burgenson, D., … Rao, G. (2019). Wood microfluidics. *Analytical Chemistry*, 91(17), 11004–11012. https://doi.org/10.1021/acs.analchem.9b01232

ASAB/ABS. (2015). Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour*, 99, 1–9. https://doi.org/10.1016/S0003-3472(14)00451-5

Avarguès-Weber, A., Lachlan, R., & Chittka, L. (2018). Bumblebee social learning can lead to suboptimal foraging choices. *Animal Behaviour*, 135, 209–214. https://doi.org/10.1016/j.anbehav.2017.11.022

Bédécarrats, A., Chen, S., Pearce, K., Cai, D., & Glanzman, D. L. (2018). RNA from trained *Aplysia* can induce an epigenetic engram for long-term sensitization in untrained *Aplysia*. *eNeuro*, 5(3). https://doi.org/10.1523/ENEURO.0038-18.2018

Belgis, Z. C., Persoone, & G., & Blaise, C. (2003). Cyst-based toxicity tests XVI––sensitivity comparison of the solid phase *Heterocypris incongruens* microbiotest with the *Hyalella azteca* and *Chironomus riparius* contact assays on freshwater sediments from Peninsula Harbour (Ontario, Canada). *Chemosphere*, 52(1), 95–101. https://doi.org/10.1016/S0045-6535(03)00186-3

Benelli, G., Romano, D., Desneux, N., Messing, R. H., & Canale, A. (2015). Sex differences in fighting-induced hyperaggression in a fly. *Animal Behaviour*, 104, 165–174. https://doi.org/10.1016/j.anbehav.2015.02.026

Bieszke, B., Namiotko, L., & Namiotko, T. (2020). Life history traits of a temporary water ostracod *Heterocypris incongruens* (Crustacea, Ostracoda) are affected by power frequency (50 Hz) electromagnetic environmental pollution. *European Zoological Journal*, 87(1), 148–155. https://doi.org/10.1080/24750263.2020.1736654

Bode, S. N. S., Adolfsson, S., Lamatsch, D. K., Martins, M. J. F., Schmit, O., Vandekerkhove, J., Mezquita, F., Namiotko, T., Rossetti, G., Schön, I., Butlin, R. K., & Martens, K. (2010). Exceptional cryptic diversity and multiple origins of parthenogenesis in a freshwater ostracod. *Molecular Phylogenetics and Evolution*, 54(2), 542–552. https://doi.org/10.1016/j.ympev.2009.08.022

Bower, G. H., & Winzenz, D. (1970). Comparison of associative learning strategies. *Psychonomic Science*, 20(2), 119–120. https://doi.org/10.3758/BF03335632

Brayner, R., Couté, A., Livage, J., Perrette, C., & Sicard, C. (2011). Micro-algal biosensors. *Analytical and Bioanalytical Chemistry*, 401(2), 581–597. https://doi.org/10.1007/s00216-011-5107-z

Briscoe, A. D., & Chittka, L. (2001). The evolution of colour vision in insects. *Annual Review of Entomology*, 46(1), 471–510. https://doi.org/10.1146/annurev.ento.46.1.471

Cafarelli, A., Verbeni, A., Poliziani, A., Dario, P., Menciassi, A., & Ricotti, L. (2017). Tuning acoustic and mechanical properties of materials for ultrasound phantoms and smart substrates for cell cultures. *Acta Biomaterialia*, 49, 368–378. https://doi.org/10.1016/j.actbio.2016.11.049

Campana, O., & Wlodkowic, D. (2018). The undiscovered country: Ecotoxicology meets microfluidics. *Sensors and Actuators B: Chemical*, 257, 692–704. https://doi.org/10.1016/j.snb.2017.11.002

Cartlidge, R., Campana, O., Nugegoda, D., & Wlodkowic, D. (2016). Optofluidic technology for monitoring rotifer *Brachionus calyciflorus* responses to regular light pulses. In M. R. Hutchinson, & E. M. Goldys (Eds.), *SPIE BioPhotonics Australasia* (Vol. 10013, p. 100132B). Adelaide, Australia: International Society for Optics and Photonics. https://doi.org/10.1117/12.2242879.

Cartlidge, R., Nugegoda, D., & Wlodkowic, D. (2017). Millifluidic Lab-on-a-Chip technology for automated toxicity tests using the marine amphipod. *Allorchestes compressa*. *Sensors and Actuators B: Chemical*, 239, 660–670. https://doi.org/10.1016/j.snb.2016.08.058

Chial, B., & Persoone, G. (2002). Cyst-based toxicity tests XIII-development of a short chronic sediment toxicity test with the ostracod crustacean *Heterocypris incongruens*: Methodology and precision. *Environmental Toxicology*, 17, 528–532. https://doi.org/10.1002/tox.10086

Chiriacò, M. S., Luvisi, A., Primiceri, E., Sabella, E., De Bellis, L., & Maruccio, G. (2018). Development of a lab-on-a-chip method for rapid assay of *Xylella fastidiosa* subsp. *pauca* strain CoDiRO. *Scientific Reports*, 8(1), 7376. https://doi.org/10.1038/s41598-018-25747-4

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Choudhury, D., van Noort, D., Iliescu, C., Zheng, B., Poon, K. L., Korzh, S., … Yu, H. (2012). Fish and chips: A microfluidic perfusion platform for monitoring zebrafish development. *Lab on a Chip, 12*(5), 892–900. https://doi.org/10.1039/C1LC20351G

Clausell-Tormos, J., Lieber, D., Baret, J. C., El-Harrak, A., Miller, O. J., Frenz, L., … Holtze, C. (2008). Droplet-based microfluidic platforms for the encapsulation and screening of mammalian cells and multicellular organisms. *Chemistry & Biology, 15*(5), 427–437. https://doi.org/10.1016/j.chembiol.2008.04.004

Crane, M. M., Chung, K., Stirman, J., & Lu, H. (2010). Microfluidics-enabled phenotyping, imaging, and screening of multicellular organisms. *Lab on a Chip, 10*(12), 1509–1517. https://doi.org/10.1039/B927258E

Davies, W. J., & Freeman, S. J. (1995). Frog embryo teratogenesis assay. In S. O'Hare, & C. K. Atterwill (Eds.), *In vitro toxicity testing protocols. Methods in molecular biology* (Vol. 43, pp. 311–316). Totowa, New Jersey, USA: Humana Press. https://doi.org/10.1385/0-89603-282-5:311.

Davis, H., & Heslop, E. (2004). Habituation of hissing by Madagascar hissing cockroaches (*Gromphadorhina portentosa*): Evidence of discrimination between humans? *Behavioural Processes, 67*(3), 539–543. https://doi.org/10.1016/j.beproc.2004.08.003

De Deckker, P. (1981). Ostracods of athalassic saline lakes. In W. D. Williams (Ed.), *Salt lakes. Developments in hydrobiology* (Vol. 5, pp. 131–144). Dordrecht: Springer. https://doi.org/10.1007/978-94-009-8665-7_1.

European Commission. (2007). *Commission recommendations of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes*. Annex II to European Council Directive 86/609. See 2007/526/EC. Retrieved from http://eurex.europa.eu/LexUriServ/LexUriServ.do?uri1/4OJ:L. 2007.197:0001:0089:EN:PDF.

Fischer, J., & Laforsch, C. (2018). The influence of gravity and light on locomotion and orientation of *Heterocypris incongruens* and *Notodromas monacha* (Crustacea, Ostracoda). *Npj Microgravity, 4*(1), 1–9. https://doi.org/10.1038/s41526-017-0037-5

Gosset, A., Ferro, Y., & Durrieu, C. (2016). Methods for evaluating the pollution impact of urban wet weather discharges on biocenosis: A review. *Water Research, 89*, 330–354. https://doi.org/10.1016/j.watres.2015.11.020

Hawkins, R. D., & Byrne, J. H. (2015). Associative learning in invertebrates. *Cold Spring Harbor Perspectives in Biology, 7*(5), a021709. https://doi.org/10.1101/cshperspect.a021709

Holmes, J. A., & Chivas, A. R. (2002). *The Ostracoda: Applications in quaternary research* (Vol. 131). Washington DC: American Geophysical Union Geophysical Monograph Series. https://doi.org/10.1029/GM131

Home, D. J., Danielopol, D. L., & Martens, K. (1998). Reproductive behaviour. In K. Martens (Ed.), *Sex and parthenogenesis; evolutionary ecology of reproductive modes in non-marine Ostracoda (Crustacea)* (pp. 157–195). Leiden: Backhuys Publishers.

Horne, D. J., Schon, I., Smith, R. J., & Martens, K. (2005). What are Ostracoda? A cladistic analysis of the extant superfamilies of the subclasses Myodocopa and Podocopa (Crustacea: Ostracoda). In S. Koenemann, & R. Jenner (Eds.), *Crustacea and arthropod relationships* (Vol. 16, pp. 249–273). Boca Raton: CRC Press. Crustacean issues.

Huang, Y., Aldasoro, C. C. R., Persoone, G., & Wlodkowic, D. (2015a). Integrated microfluidic technology for sub-lethal and behavioural marine ecotoxicity biotests. In *Bio-MEMS and medical microdevices II* (Vol. 9518, p. 95180F). International Society for Optics and Photonics. https://doi.org/10.1117/12.2180692.

Huang, Y., Nigam, A., Campana, O., Nugegoda, D., & Wlodkowic, D. (2016b). Miniaturized video-microscopy system for near real-time water quality biomonitoring using microfluidic chip-based devices. In *SPIE BioPhotonics Australasia* (Vol. 10013, p. 100131R). International Society for Optics and Photonics. https://doi.org/10.1117/12.2242826.

Huang, Y., Nugegoda, D., & Wlodkowic, D. (2015b). Automation of Daphtoxkit-F biotest using a microfluidic Lab-on-a-Chip technology. In *Micro+ nano materials, devices, and systems* (Vol. 9668, p. 966813). International Society for Optics and Photonics. https://doi.org/10.1117/12.2202396.

Huang, Y., Persoone, G., Nugegoda, D., & Wlodkowic, D. (2016a). Enabling sub-lethal behavioural ecotoxicity biotests using microfluidic Lab-on-a-Chip technology. *Sensors and Actuators B: Chemical, 226*, 289–298. https://doi.org/10.1016/j.snb.2015.11.128

Illing, R., Burkart, C., Pfitzner, D., Jungmann, D., Baraban, L., & Cuniberti, G. (2016). Ecotoxicity assessment using ciliate cells in millifluidic droplets. *Biomicrofluidics, 10*(2), Article 024115. https://doi.org/10.1063/1.4944869

Jung, J., Nakajima, M., Masaru, T., Huang, Q., & Fukuda, T. (2014). A microfluidic device with multi-valves system to enable several simultaneous exposure tests on *Caenorhabditis elegans*. *Journal of Micromechanics and Microengineering, 24*(3), Article 035012. https://doi.org/10.1088/0960-1317/24/3/035012

Jung, J., Nakajima, M., Tajima, H., Huang, Q., & Fukuda, T. (2013). A microfluidic device for the continuous culture and analysis of *Caenorhabditis elegans* in a toxic aqueous environment. *Journal of Micromechanics and Microengineering, 23*(8), Article 085008. https://doi.org/10.1088/0960-1317/23/8/085008

Kim, B. C., & Gu, M. B. (2003). A bioluminescent sensor for high throughput toxicity classification. *Biosensors and Bioelectronics, 18*(8), 1015–1021. https://doi.org/10.1016/S0956-5663(02)00220-8

Kim, J. H., Lee, S. H., Cha, Y. J., Hong, S. J., Chung, S. K., Park, T. H., & Choi, S. S. (2017). *C. elegans*-on-a-chip for in situ and in vivo Ag nanoparticles' uptake and toxicity assay. *Scientific Reports, 7*, 40225. https://doi.org/10.1038/srep40225

Kim, Y., Mo, H. H., Son, J., Lee, Y. S., Lee, S. E., & Cho, K. (2015). Interactive effects of water pH and hardness levels on the growth and reproduction of *Heterocypris incongruens* (Crustacea: Ostracoda). *Hydrobiologia, 753*(1), 97–109. https://doi.org/10.1007/s10750-015-2199-z

Kohler, S. L., & McPeek, M. A. (1989). Predation risk and the foraging behaviour of competing stream insects. *Ecology, 70*(6), 1811–1825. https://doi.org/10.2307/1938114

Krasne, F. B. (1973). Learning in Crustacea. In W. C. Corning, J. A. Dyal, & A. O. D. Willows (Eds.), *Invertebrate learning* (Vol. 2, pp. 49–130). New York: Plenum. Arthropods and gastropod mollusks.

Lafleur, J. P., Jönsson, A., Senkbeil, S., & Kutter, J. P. (2016). Recent advances in lab-on-a-chip for biosensing applications. *Biosensors and Bioelectronics, 76*, 213–233. https://doi.org/10.1016/j.bios.2015.08.003

Lammer, E., Carr, G. J., Wendler, K., Rawlings, J. M., Belanger, S. E., & Braunbeck, T. (2009). Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology, 149*(2), 196–209. https://doi.org/10.1016/j.cbpc.2008.11.006

Laut, L. L. M., Clemente, I. M. M. M., Belart, P., Martins, M. V. A., Frontalini, F., Laut, V. M., … da Conceição Rodrigues, M. A. (2016). Multiproxies (benthic foraminifera, ostracods and biopolymers) approach applied to identify the environmental partitioning of the Guadiana River Estuary (Iberian Peninsula). *Journal of Sedimentary Environments, 1*(2), 178–195. https://doi.org/10.12957/jse.2016.22534

Lawrence, J. R., Scharf, B., Packroff, G., & Neu, T. R. (2002). Microscale evaluation of the effects of grazing by invertebrates with contrasting feeding modes on river biofilm architecture and composition. *Microbial Ecology, 44*(3), 199–207. https://doi.org/10.1007/s00248-001-1064-y

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Lee, S. A., Zheng, G., Mukherjee, N., & Yang, C. (2012). On-chip continuous monitoring of motile microorganisms on an ePetri platform. *Lab on a Chip, 12*(13), 2385–2390. https://doi.org/10.1039/C2LC40090A

Manfredi, L., Assaf, T., Mintchev, S., Marrazza, S., Capantini, L., Orofino, S., … Stefanini, C. (2013). A bioinspired autonomous swimming robot as a tool for studying goal-directed locomotion. *Biological Cybernetics, 107*(5), 513–527. https://doi.org/10.1007/s00422-013-0566-2

Manz, A., Graber, N., & Widmer, H.Á. (1990). Miniaturized total chemical analysis systems: A novel concept for chemical sensing. *Sensors and Actuators B: Chemical, 1*(1–6), 244–248. https://doi.org/10.1016/0925-4005(90)80209-I

Martens, K., Schön, I., Meisch, C., & Horne, D. J. (2008). Global diversity of ostracods (Ostracoda, Crustacea) in freshwater. *Hydrobiologia, 595*, 185–193. https://doi.org/10.1007/s10750-007-9245-4

Mather, J. A. (2008). Cephalopod consciousness: Behavioural evidence. *Consciousness and Cognition, 17*(1), 37–48. https://doi.org/10.1016/j.concog.2006.11.006

Mather, J. A., & Kuba, M. J. (2013). The cephalopod specialties: Complex nervous system, learning, and cognition. *Canadian Journal of Zoology, 91*(6), 431–449. https://doi.org/10.1139/cjz-2013-0009

Mbahinzireki, G., Uiblein, F., & Winkler, H. (1991). Microhabitat selection of ostracods in relation to predation and food. *Hydrobiologia, 222*(2), 115–119. https://doi.org/10.1007/BF00006099

McGuire, T. R. (1984). Learning in three species of diptera: The blow fly *Phormia regina*, the fruit fly *Drosophila melanogaster*, and the house fly *Musca domestica*. *Behavior Genetics, 14*(5), 479–526. https://doi.org/10.1007/BF01065445

McKenzie, K. G. (1972). Contribution to the ontogeny and phylogeny of Ostracoda. *Proceedings of the International Paleontological Union, the XXIII International Geological Congress, 1968*, 165–188.

Meisch, C. (2000). *Freshwater Ostracoda from Western and central Europe. Süßwasserfauna von Mitteleuropa, 8/3*. Heidelberg: Spektrum Akademischer Verlag.

Menzel, R., Brembs, B., & Giurfa, M. (2007). Cognition in invertebrates. In J. H. Kaas (Ed.), *Evolution of nervous systems* (Vol. II, pp. 403–422). Oxford: Academic Press. Evolution of nervous systems in invertebrates.

Mesquita-Joanes, F., Smith, A. J., & Viehberg, F. A. (2012). The ecology of Ostracoda across levels of biological organisation from individual to ecosystem: A review of recent developments and future potential. In D. J. Horne, J. Holmes, J. Rodriguez-Lazaro, & F. A. Viehberg (Eds.), *Ostracoda as proxies for quaternary climate change* (pp. 15–35). Amsterdam: Elsevier.

Miličić, D. M., Majstorović, A. P., Pavković-Lučić, S. B., & Savić, T. T. (2015). Behaviour and food selection of *Heterocypris incongruens* (Ostracoda). *Crustaceana, 88*(10–11), 1097–1110. https://doi.org/10.1163/15685403-00003479

Mills, C. L., Shukla, D. H., & Compton, G. J. (2006). Development of a new low cost high sensitivity system for behavioural ecotoxicity testing. *Aquatic Toxicology, 77*(2), 197–201. https://doi.org/10.1016/j.aquatox.2005.12.003

Moroz, L. L. (2011). Aplysia. *Current Biology, 21*(2), R60. https://doi.org/10.1016/j.cub.2010.11.028

Muna, M., Blinova, I., Kahru, A., Vinković Vrček, I., Pem, B., Orupõld, K., & Heinlaan, M. (2019). Combined effects of test media and dietary algae on the toxicity of CuO and ZnO nanoparticles to freshwater microcrustaceans *Daphnia magna* and *Heterocypris incongruens*: Food for thought. *Nanomaterials, 9*(1), 23. https://doi.org/10.3390/nano9010023

Nargeot, R., & Bédécarrats, A. (2017). *Associative learning in invertebrates. The oxford handbook of invertebrate neurobiology*. Oxford: Oxford University Press.

Oakley, T. H. (2005). Myodocopa (Crustacea: Ostracoda) as models for evolutionary studies of light and vision: Multiple origins of bioluminescence and extreme sexual dimorphism. *Hydrobiologia, 538*(1–3), 179–192. https://doi.org/10.1007/s10750-004-4961-5

Oakley, T. H., & Huber, D. R. (2004). Differential expression of duplicated opsin genes in two eyetypes of ostracod crustaceans. *Journal of Molecular Evolution, 59*(2), 239–249. https://doi.org/10.1007/s00239-004-2618-7

Oakley, T. H., Wolfe, J. M., Lindgren, A. R., & Zaharoff, A. K. (2013). Phylotranscriptomics to bring the understudied into the fold: Monophyletic Ostracoda, fossil placement, and pancrustacean phylogeny. *Molecular Biology and Evolution, 30*(1), 215–233. https://doi.org/10.1093/molbev/mss216

Osorio, D., & Bacon, J. P. (1994). A good eye for arthropod evolution. *BioEssays, 16*(6), 419–424. https://doi.org/10.1002/bies.950160610

Pane, L., Agrone, C., Giacco, E.,, Somà, A., & Mariottini, G. (2012). Utilization of marine crustaceans as study models: A new approach in marine ecotoxicology for European (REACH) regulation. *Ecotoxicology, 5*, 91–106. https://doi.org/10.5772/28513

Parker, A. R. (1995). Discovery of functional iridescence and its coevolution with eyes in the phylogeny of Ostracoda (Crustacea). *Proceedings of the Royal Society of London. Series B: Biological Sciences, 262*(1365), 349–355. https://doi.org/10.1098/rspb.1995.0216

Perry, C. J., Barron, A. B., & Cheng, K. (2013). Invertebrate learning and cognition: Relating phenomena to neural substrate. *Wiley Interdisciplinary Reviews: Cognitive Science, 4*(5), 561–582. https://doi.org/10.1002/wcs.1248

Pieri, V., Marrone, F., Martens, K., & Rossetti, G. (2020). An updated checklist of Recent ostracods (Crustacea: Ostracoda) from inland waters of Sicily and adjacent small islands with notes on their distribution and ecology. *The European Zoological Journal, 87*(1), 714–740. https://doi.org/10.1080/24750263.2020.1839581

Pieri, V., Vandekerkhove, J., & Goi, D. (2012). Ostracoda (Crustacea) as indicators for surface water quality: A case study from the Ledra river basin (NE Italy). *Hydrobiologia, 688*(1), 25–35. https://doi.org/10.1007/s10750-010-0568-1

R Development Core Team. (2019). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rapp, H., Nawrot, M. P., & Stern, M. (2020). Numerical cognition based on precise counting with a single spiking neuron. *IScience, 23*(2), 100852. https://doi.org/10.1016/j.isci.2020.100852

Reaka, M. L. (1980). On learning and living in holes by mantis shrimp. *Animal Behaviour, 28*(1), 111–115. https://doi.org/10.1016/S0003-3472(80)80014-5

Roca, J. R., Baltanás, A., & Uiblein, F. (1993). Adaptive responses in *Cypridopsis vidua* (Crustacea: Ostracoda) to food and shelter offered by a macrophyte (*Chara fragilis*). *Hydrobiologia, 262*(2), 127–131. https://doi.org/10.1007/BF00007513

Romano, D., Benelli, G., Hwang, J. S., & Stefanini, C. (2019b). Fighting fish love robots: Mate discrimination in males of a highly territorial fish by using female-mimicking robotic cues. *Hydrobiologia, 833*(1), 185–196. https://doi.org/10.1007/s10750-019-3899-6

Romano, D., Benelli, G., Stefanini, C., Desneux, N., Ramirez-Romero, R., Canale, A., & Lucchi, A. (2018). Behavioural asymmetries in the mealybug parasitoid *Anagyrus* sp. near *pseudococci*: Does lateralized antennal tapping predict male mating success? *Journal of Pest Science, 91*(1), 341–349. https://doi.org/10.1007/s10340-017-0903-7

Romano, D., Donati, E., Benelli, G., & Stefanini, C. (2019a). A review on animal–robot interaction: From bio-hybrid organisms to mixed societies. *Biological Cybernetics, 113*(3), 201–225. https://doi.org/10.1007/s00422-018-0787-5

Please cite this article as: Romano, D et al., Learning on a chip: Towards the development of trainable biohybrid sensors by investigating cognitive processes in non-marine Ostracoda via a miniaturised analytical system, *Biosystems Engineering*, https://doi.org/10.1016/j.biosystemseng.2021.11.004

Romano, D., & Stefanini, C. (2021). Individual neon tetras (*Paracheirodon innesi*, Myers) optimise their position in the group depending on external selective contexts: Lesson learned from a fish-robot hybrid school. *Biosystems Engineering, 204*, 170–180. https://doi.org/10.1016/j.biosystemseng.2021.01.021

Rossi, V., Benassi, G., Belletti, F., & Menozzi, P. (2011). Colonization, population dynamics, predatory behaviour and cannibalism in *Heterocypris incongruens* (Crustacea: Ostracoda). *Journal of Limnology, 70*(1), 102–108. https://doi.org/10.3274/JL11-70-1-12

Rossi, V., & Menozzi, P. (1993). The clonal ecology of *Heterocypris incongruens* (Ostracoda): Life-history traits and photoperiod. *Functional Ecology, 7*, 177–182.

Ruiz, F., Abad, M., Bodergat, A. M., Carbonel, P., Rodríguez-Lázaro, J., González-Regalado, M. L., … Prenda, J. (2013). Freshwater ostracods as environmental tracers. *International journal of Environmental Science and Technology, 10*(5), 1115–1128. https://doi.org/10.1007/s13762-013-0249-5

Schön, I., Martens, K., Van Doninck, K., & Butlin, R. K. (2003). Evolution in the slow lane: Molecular rates of evolution in sexual and asexual ostracods (Crustacea: Ostracoda). *Biological Journal of the Linnean Society, 79*(1), 93–100. https://doi.org/10.1046/j.1095-8312.2003.00186.x

Schön, I., Pieri, V., Sherbakov, D. Y., & Martens, K. (2017). Cryptic diversity and speciation in endemic *cytherissa* (Ostracoda, Crustacea) from lake Baikal. *Hydrobiologia, 800*(1), 61–79. https://doi.org/10.1007/s10750-017-3259-3

Schön, I., Pinto, R. L., Halse, S., Smith, A. J., Martens, K., & Birky, C. W., Jr. (2012). Cryptic species in putative ancient asexual darwinulids (Crustacea, Ostracoda). *PLoS One, 7*(7), Article e39844. https://doi.org/10.1371/journal.pone.0039844

Shahid, M. T., Khan, M. A., & Khan, M. Z. (2019). Design and development of a computer numeric controlled 3D printer, laser cutter and 2D plotter all in one machine. In *16th international bhurban conference on applied sciences and technology (IBCAST)* (pp. 569–575). IEEE. https://doi.org/10.1109/IBCAST.2019.8667138.

Shanti, A., Teo, J., & Stefanini, C. (2018). In vitro immune organs-on-chip for drug development: A review. *Pharmaceutics, 10*(4), 278. https://doi.org/10.3390/pharmaceutics10040278

Shigeno, S., Andrews, P. L., Ponte, G., & Fiorito, G. (2018). Cephalopod brains: An overview of current knowledge to facilitate comparison with vertebrates. *Frontiers in Physiology, 9*, 952. https://doi.org/10.3389/fphys.2018.00952

Sinha, A., Gopinathan, P., Chung, Y. D., Shiesh, S. C., & Lee, G. B. (2019). Simultaneous detection of multiple NT-proBNP clinical samples utilizing an aptamer-based sandwich assay on an integrated microfluidic system. *Lab on a Chip, 19*(9), 1676–1685. https://doi.org/10.1039/C9LC00115H

Siveter, D. J., & Vannier, J. M. (1990). The silurian myodocope ostracode *entomozoe* from the pentland hills, scotland: Its taxonomic, ecological and phylogenetic significance and the affinity of the bolbozoid myodocopes. *Earth and Environmental Science Transactions of The Royal Society of Edinburgh, 81*(1), 45–67. https://doi.org/10.1017/S0263593300005125

Smith, A. J., & Horne, D. J. (2002). *Ecology of marine, marginal marine and nonmarine ostracodes* (Vol. 131, pp. 37–64). *Washington DC American Geophysical Union Geophysical Monograph Series*.

Smith, A. J., Horne, D. J., Martens, K., & Schön, I. (2015). Class Ostracoda. In J. Thorp, & D. C. Rogers (Eds.), *Ecology and general biology: Thorp and covich's freshwater invertebrates* (pp. 757–780). Amsterdam: Academic Press.

Smith, R. J., & Matzke-Karasz, R. (2008). The organ on the first segment of the cypridoidean (Ostracoda, Crustacea) antennule: Morphology and phylogenetic significance. *Senckenbergiana Lethaea, 88*(1), 127–140. https://doi.org/10.1007/BF03043984

Tanaka, G. (2005). Morphological design and fossil record of the podocopid ostracod naupliar eye. *Hydrobiologia, 538*(1–3), 231–242. https://doi.org/10.1007/s10750-004-4969-x

Tanaka, G. (2006). Functional morphology and light-gathering ability of podocopid ostracod eyes and the palaeontological implications. *Zoological Journal of the Linnean Society, 147*(1), 97–108. https://doi.org/10.1111/j.1096-3642.2006.00216.x

Temiz, Y., Lovchik, R. D., Kaigala, G. V., & Delamarche, E. (2015). Lab-on-a-chip devices: How to close and plug the lab? *Microelectronic Engineering, 132*, 156–175. https://doi.org/10.1016/j.mee.2014.10.013

Uiblein, F., Eberstaller, J., Pöckl, M., & Winkler, H. (1992). Effects of differential prey mobility on the foraging behaviour of a cyprinid fish, *Vimba elongata*. *Ethology Ecology & Evolution, 4*(3), 293–297. https://doi.org/10.1080/08927014.1992.9523140

Uiblein, F., Roca, J. R., Baltanás, A., & Danielopol, D. L. (1996). Tradeoff between foraging and antipredator behaviour in a macrophyte dwelling ostracod. *Archiv für Hydrobiologie, 137*(1), 119–133.

Vannier, J., Abe, K., & Ikuta, K. (1998). Feeding in myodocopid ostracods: Functional morphology and laboratory observations from videos. *Marine Biology, 132*(3), 391–408. https://doi.org/10.1007/s002270050406

Vasile, M. J., Nassar, R., Xie, J., & Guo, H. (1999). Microfabrication techniques using focused ion beams and emergent applications. *Micron, 30*(3), 235–244. https://doi.org/10.1016/S0968-4328(99)00008-6

Verslycke, T., Ghekiere, A., Raimondo, S., & Janssen, C. (2007). Mysid crustaceans as standard models for the screening and testing of endocrine-disrupting chemicals. *Ecotoxicology, 16*(1), 205. https://doi.org/10.1007/s10646-006-0122-0

Wakayama, N. (2007). Embryonic development clarifies polyphyly in ostracod crustaceans. *Journal of Zoology, 273*(4), 406–413. https://doi.org/10.1111/j.1469-7998.2007.00344.x

Wilkinson, I., Wilby, P., Williams, P., Siveter, D., & Vannier, J. (2007). Ostracod carnivory through time. In A. M. T. Elewa (Ed.), *Predation in organisms: A distinct phenomenon* (pp. 39–57). Heidelberg: Springer-Verlag.

Williams, M., Siveter, D. J., Salas, M. J., Vannier, J., Popov, L. E., & Pour, M. G. (2008). The earliest ostracods: The geological evidence. *Senckenbergiana Lethaea, 88*(1), 11–21. https://doi.org/10.1007/BF03043974

Won, E. J., Han, J., Kim, D. H., Dahms, H. U., & Lee, J. S. (2017). Rotifers in ecotoxicology. In A. Hagiwara, & T. Yoshinaga (Eds.), Rotifers *(pp. 149-176). Fisheries science series*. Singapore: Springer. https://doi.org/10.1007/978-981-10-5635-2_10.

Ydenberg, R. C., & Dill, L. M. (1986). The economics of fleeing from predators. *Advances in the Study of Behavior, 16*(C), 229–249. https://doi.org/10.1016/S0065-3454(08)60192-8

Yoo, S. K., Lee, J. H., Yun, S. S., Gu, M. B., & Lee, J. H. (2007). Fabrication of a bio-MEMS based cell-chip for toxicity monitoring. *Biosensors and Bioelectronics, 22*(8), 1586–1592. https://doi.org/10.1016/j.bios.2006.07.014

Zabihihesari, A., Hilliker, A. J., & Rezai, P. (2019). Fly-on-a-Chip: Microfluidics for *Drosophila melanogaster* studies. *Integrative Biology, 11*(12), 425–443. https://doi.org/10.1093/intbio/zyz037

Zhang, B., Li, Y., He, Q., Qin, J., Yu, Y., Li, X., … Chen, Z. (2014). Microfluidic platform integrated with worm-counting setup for assessing manganese toxicity. *Biomicrofluidics, 8*(5), Article 054110. https://doi.org/10.1063/1.4896663

Zylinski, S. (2015). Fun and play in invertebrates. *Current Biology, 25*(1), R10–R12. https://doi.org/10.1016/j.cub.2014.09.068

Please cite this article as: Romano, D et al., Learning on a chip: Towards the development of trainable biohybrid sensors by investigating cognitive processes in non-marine Ostracoda via a miniaturised analytical system, *Biosystems Engineering*, https://doi.org/10.1016/j.biosystemseng.2021.11.004