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A comparison of hatchery-rearing in exercise to wild animal physiology and reflex behavior in *Aplysia californica*

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ABSTRACT

Aplysia californica was hatchery-reared in two turbulence protocols intended to imitate the intermittent turbulence of the native habitat and to promote development of the foot muscle from the exercise of adhering to the substrate. Hatchery-reared animals in turbulence regimes were compared to siblings reared in quiet water, and to wild animals, using noninvasive assessments of the development of the foot muscle. The objective was to learn if the turbulence-reared phenotype mimicked laboratory-targeted aspects of the wild phenotype, that is, reflex behavior, swim tunnel performance, and resting oxygen consumption (MO_2). No group exhibited different MO_2. MO_2 values for all of the compared groups of animals followed the power law, with an exponent of 0.69, consistent with this relationship throughout the animal kingdom. Turbulence-induced exercise did not affect the righting reflex or the tail withdrawal reflex, standard behavioral tests that involve the foot muscle, compared to quiet water-reared siblings. Wild individuals had significantly shorter time-to-right than all hatchery reared animals, however, wild animals did not perform better in flume tests. That turbulence-reared hatchery- or wild animals lacked superior flume performance suggests that this species may shelter from intertidal wave energy to remain near its optimal feeding areas.

1. Introduction

The marine invertebrate, *Aplysia californica* (Aplysia) is renowned as a model of the nervous system due to its advantages of a simple, well-mapped brain and capabilities for learning. The National Resource for Aplysia at the University of Miami in Miami, Florida, USA, has > 25 years' experience growing this opisthobranch species for the scientific community (Capo et al., 2002, 2003; Stommes et al., 2005). The normal habitat for this species is the rocky intertidal of the North American Pacific coast, where their red algal food is abundant (Audesirk, 1979; Carefoot, 1987). Wave energy in this environment likely promotes isometric contractions, particularly in foot muscle of Aplysia, during adherence to the rock substrate. This is a form of exercise that is absent in rearing conditions at the Resource. There, a gentle flow of fresh seawater with no additional sources of water movement produces less water energy, and thus would promote less isometric exercise than would the rocky intertidal. As a result of these differences, muscle mass during somatic growth, as well as sensory stimulation, may differ between hatchery and wild conditions. The goal of experiments summarized here was to estimate the difference the environment might make in the outcome of common neurophysiological protocols used on this animal model.

Isometric contractions in human skeletal muscles elicit the activity of motoneurons during muscle training, especially at the beginning of a strength improvement program (reviewed in Griffin and Cafarelli, 2005, Folland and Williams, 2007). The effect is believed to be due, in part, to enhanced recruitment of muscle fibers to the task via enhanced muscle fiber responses to neural stimuli. Rearing Aplysia in conditions that stimulate isometric contraction of the foot muscles similarly may affect the central nervous system. If so, the neural stimulation provided by isometric exercise could impact learning paradigms in which these animals are regularly used.

Examples in which muscle actions can recruit neurons or modulate neuronal firing patterns as part of the initiation or modulation of motor programs, respectively, exist in Aplysia. The neurophysiological control and modulation of the Aplysia buccal smooth muscle model is well studied, with many neural influences described (Weiss et al., 1978; Hurwitz et al., 2003; Brezina et al., 2003). Foot muscle neuromotor

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Abbreviations: MO_2, resting oxygen consumption; TTR, time-to-right; TWR, tail withdrawal reflex

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patterns focused on locomotion (Fredman and Jahan-Parwar, 1980; Jahan-Parwar and Fredman, 1979; McPherson and Blankenship, 1992; Bruno et al., 2017) have also received attention. These studies were centered on neuromuscular control that changes the length of muscles, rather than isometric contractions. Finally, there is evidence that some aspect of a turbulent water location within its normal habitat might influence how Aplysia respond to tactile stimuli. Carew and Kupfermann (1974) recorded that animals found in areas described as turbulent were less responsive to initial and repeated siphon withdrawal stimuli, intended to evoke habituation, than individuals found in quiet water tide pools, as though they were already habituated to tactile stimuli. These examples provide evidence that muscle activity and sensory experience provided by the local environment can influence neural activity and behavior in this model gastropod.

Aplysia were reared in conditions intended to imitate the turbulence of the rocky shoreline to begin to address the effects on foot muscle development of the routine exercise of adhering firmly to the substrate. We tested for indices of positive foot muscle development non-invasively, by challenging animals to perform at steadily increasing speeds in a swim tunnel and by measuring oxygen consumption. We monitored reflex behaviors that involve foot muscle, to learn if exercise affected their execution.

2. Materials and methods

2.1. Husbandry and turbulence rearing regimes

Animals from a single cohort spawned from wild-caught brood stock were reared at ~100 individuals per 16 L cage starting several weeks post-metamorphosis and fed standard ration (< ad lib.) *Agardhiella subulata* until age 4 months and ~5 g. Animals were placed at a density of 10/cage into the exercise protocols illustrated in Fig. 1, and fed nominally ad lib., which provided satiation rations over 4 h with little uneaten food. Three cages were set up for controls and for each of 2 exercise regimes such that $n = 30$ for each treatment. All these animals are referred to as cohort animals.

Cohort animals were reared under 3 different protocols. Fresh, 15 °C seawater flowed into cages at ~2 L per minute from a corner valve in the still water controls (Fig. 1A), or at ~1 L per minute flow from the corner valve, plus intermittent turbulent flow from either electric pumps or dumpbuckets (Fig. 1B and C). All cage bottoms were perforated with 6 discontinuous, 0.5 cm slits along the 43 cm long axis, and cages were suspended in fiberglass reservoirs to allow single-pass seawater flow. Each turbulent flow protocol, pumps or dumpbuckets, was intended to deliver approximately the same volume of fresh seawater flow as for control cages during each hr. For the pump exercise program, seawater was delivered to each pump cage during a 30-s period at 2.8 L/min, every 5 min, from a 2400 gal/h capacity aquarium pump set into a ~400-L reservoir located below the cages. Six, ~3-cm aperture duckbill valves placed around the inside perimeter of each rectangular rearing cage created chaotic turbulence from the pump flow, and minimized cage areas without flow (Fig. 1B, showing small animals with flared parapodia as the flow dislodged and moved them). For the dumpbucket exercise program, seawater was delivered to each dumpbucket cage via a ~3 cm diameter PVC pipe attached to a 20-L bucket suspended above the cage, that filled during the non-dump period, and discharged during ~35 s every ~7.5 min (Fig. 1C).

2.2. Assessment schedule

The assessment scheme is shown in Fig. 2. At one month in the experiment, when the cohort animals were ~age 5 months and ~19 g (Fig. 2, row 2), mass and seawater volume displacement measurements were made on them all to estimate density. Reflex behaviors that involve the foot muscle exercised in dumpbucket and pumps exercise were assessed on 12 cohort animals haphazardly chosen from each exercise regime and still-water controls, alongside 14 wild-caught animals of unknown age that arrived in the hatchery that week, mass ~120 g. Single trials for righting (time-to-right, TTR) and tail withdrawal reflexes (TWR) were executed as in Kempsell and Fieber (2014). Flume tests for muscle performance were executed on these 36 cohort animals and on 12 of the wild animals, either the same day or the following day.

Five weeks later, at ~2 months in the experiment, when the cohort animals were age ~6 months and ~140 g (Fig. 2, row 3), morphometric and behavioral assessments were made on all cohort animals in the experiment. Flume test assessments were made on 12 cohort animals chosen haphazardly from controls and each exercise regime, alongside 12 recently wild-caught animals of unknown age but similar mass as the hatchery animals. Oxygen consumption (MO_2) was measured in 11 of these cohort animals following their flume tests, and in 5 of the wild animals. The cohort animals reached sexual maturity 2 d prior to these final tests. Exercise rearing ceased, and all cohort animals were returned to still-water rearing but maintained as distinct groups.

Three weeks later, at age ~7 months and ~230 g (Fig. 2, row 4), morphometric and TWR measurements were made on 8 cohort animals that had been in each treatment, but that had been maintained in still water rearing conditions since the 2 month assessments.

Four months later, when the hatchery cohort was age ~11 months and ~300 g (Fig. 2, row 5), morphometric, behavioral, muscle performance and MO_2 measurements were made on 12, still water-reared (control) animals. Reflex behavior assessments indicated these animals had reached aging stage AII (Kempsell and Fieber, 2014).

2.3. Muscle performance flume test protocols

Individual flume tests assessing the ability of the animals to adhere to a smooth surface were executed three times during the experiment, at times that corresponded to the morphometric and behavioral measurements (TTR, TWR) described above. All flume tests were conducted at 16 °C. At one month in the experiment (Fig. 2, row 2), 12 cohort animals from the still-water control and each turbulence treatment, and 12 wild animals, were placed one at a time into 5.3-L Brett-type swim chamber respirometers (Loligo Systems ApS). The working section of each of the 3 identical respirometers measured $28 \times 7 \times 7$ cm. The flume speed protocol began with orientation of the animal in the center of the flume chamber at a flow speed of 5 cm/s until it adhered to the chamber bottom, then the flume chamber lid was attached. The test consisted of 1 min at, consecutively, 10, 19, 25, 30, 35, 40, 45 cm/s, and finally 4.5 min at 50 cm/s. The protocol ran until the animal either lost contact with the bottom and was pinned by the flow to the rear wall of the chamber, or the protocol was completed (11.5 min). If the animal completed the protocol without losing its hold on the chamber bottom, it was considered a no-failure trial. Fluming experimenters were unaware of the treatment group or wild identity of animals studied.

Five weeks later, when the cohort animals were age ~6 months (Fig. 2, row 3), fluming exercise was repeated for a haphazardly chosen 12 cohort animals per treatment and 12 wild animals. Experimenters were blind to the group. Orientation of the animal in the center of the flume chamber at a flow speed of 20 cm/s adhered it to the bottom, then the flume lid was attached. The 14 min protocol was 2 min at, consecutively, 20, 35, 50, 65, 80 cm/s, and 4 min at 95 cm/s, or until the animal lost contact with the bottom and was pinned by the flow to the rear wall of the chamber. If the animal reached 14 min, water velocity was increased to 100 cm/s and held at this speed until failure. This fluming protocol was repeated on aged II, still water controls ~5 months later (Fig. 2, row 5).

2.4. Respirometry protocol

MO_2 (mg O_2/kg/h, or mg O_2/h) measurements followed the age 6 months flume muscle performance trials (Fig. 2, row 3). A subset of