

Presence of Gamma-Aminobutyric Acid (Gaba) in the Pedal Mucus of the Critically Endangered Species *Patella ferruginea*

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Abstract *Patella ferruginea* is a giant patellid limpet endemic to the Mediterranean Sea. It presently is in danger of extinction, and many have called for developing conservation measures including the mass production of spats for re-introduction projects. However, so far all attempts have been relatively unsuccessful. Previous work analyzing the effects of gamma-aminobutyric acid (GABA) on the recruitment of patellid limpets has shown that larvae respond to the presence of this signaling molecule. This response could explain the gregarious distribution typical of this species. In the present study, we demonstrated that GABA is naturally secreted by *P. ferruginea* in the pedal mucus. GABA is preferentially secreted during the summer, coinciding with the reproductive resting period of the species. Further research should aim to analyze the effects of GABA on larval development and metamorphosis in order to assess its potential use to improve conservation efforts.

Keywords Chemical cues · Limpet · Mucus · Recruitment

Introduction

Settlement by active habitat selection is common in nature and may involve chemical cues produced by conspecifics that attract the larvae to favorable conditions (e.g., Pawlik 1992). A variety of chemicals can act as signals to induce metamorphosis, including gamma-aminobutyric acid (GABA), which has been identified as an important signal compound in gastropods (e.g., García-Lavandeira et al. 2005; Laimek et al. 2008; Rivera-Ingraham et al. 2011).

The ferruginean limpet (*Patella ferruginea* Gmelin, 1791) is the most endangered macroinvertebrate in the western Mediterranean, and the reinforcement of populations and/or reintroduction of specimens to areas where the species is in danger of extinction has been proposed as a conservational tool (MMAMRM 2008). Rivera-Ingraham et al. (2011) examined whether GABA enhanced the recruitment of several limpet species (including *P. ferruginea*) to artificial plates used as units for reintroduction. The study highlighted how the application of GABA in intertidal areas enhanced recruitment of limpet larvae and caused settlement to start earlier than in controls. Under natural conditions *P. ferruginea* settles gregariously. GABA had effects on the settlement of larvae similar to the presence of adult conspecifics (Rivera-Ingraham et al. 2011), thus indicating that the adults may be a source of signaling chemicals that stimulate settlement. In this study, we aimed to determine if GABA is naturally present in the pedal mucus of *P. ferruginea* and to detect possible changes related to size and reproductive cycle.

Methods and Materials

The study was conducted in Ceuta, Strait of Gibraltar, home to one of the most important remaining populations of

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P. ferruginea and also populated by *Cymbula nigra*. Limpets were collected from an area known as “Parque del Mediterráneo”, inaccessible to the public and thereby housing major populations of these two species.

We used a group size of 18 *P. ferruginea* (9 males and 9 females; average size=6.73±1.44 cm and 8.22±0.65 cm, respectively) and 6 *C. nigra* limpets (3 males and 3 females; average size=7.4±0.11 cm and 7.83±0.20 cm, respectively) serving as controls. In 2012, coinciding with the reproductive period of *P. ferruginea* (December) (Frenkiel 1975), animals were removed carefully from the rock with a spatula during low tide. Mucus samples were taken by allowing limpets to move for 30 min over the surface of a glass petri dish, big enough to allow the animal to move and leave a trail of pedal mucus (Donovan and Carefoot 1997). Animals were marked individually with an epoxy tag (as in Espinosa et al. 2008) and were then returned to their home scars, ensuring that they fully re-adhered to avoid their loss.

Previously marked limpets were retrieved in July 2013 (14 out of 18), coinciding with the reproductive resting period of *P. ferruginea*, and were subjected to the same procedure.

Petri dishes containing mucus samples were allowed to dry at RT for 48 hr. The dry surface was scraped with a sterilized razor blade, and the mucus sample was weighed and preserved at -20 °C until further analyses.

Samples were diluted in 200–400 µl MilliQ water and intensively vortexed. Mass spectral measurements to quantify GABA content were performed on a triple quadrupole mass spectrometer (API 4000 QTrap, AB Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray interface, coupled to a liquid chromatograph (model 1100, Agilent, Waldbronn, Germany). The liquid chromatograph includes a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A). Mass spectrometric analyses for paralytic shellfish toxins (PSTs) were performed on a hydrophilic interaction liquid ion chromatography (HILIC) stationary phase. The analytical column (150×4.6 mm) was packed with 5 µm ZIC-HILIC (Merck, Darmstadt, Germany) and maintained at 35 °C. The flow rate was 0.7 ml min⁻¹ and gradient elution performed with eluent A (2 mM formic acid and 5 mM ammonium formate in acetonitrile in water (80:20 v/v)) and eluent B (10 mM formic acid and 10 mM ammonium formate in water). The gradient process involved: 20 min column equilibration using 80 % eluent A solution, linear gradient for 5 min reaching 65 % eluent A, 10 min for a 40 % eluent A concentration, and one min for returning to initial conditions (total run time: 36 min).

Selected reaction monitoring experiments were performed in the positive ion mode. Three transitions were used: *m/z* 104>87 (for quantification), 104>86 and 104>69 (for quality controls). Dwell times of 20 ms were used for each transition. The source parameters were: curtain gas (10 psi), temperature

(550 °C), ion spray voltage (5500 V), gas 1 (30 psi), gas 2 (40 psi), interface heater (off), collision gas (medium), declustering potential (26 V), entrance potential (10 V), collision cell exit potential (12 V), and collision energies (15 V for the transitions *m/z* 104>87 and 104>86, whereas 45 V for *m/z* 104>69). Samples were calibrated against an external three point curve consisting of GABA standards of 20, 200, and 1000 pg µl⁻¹. The limit of detection (LOD) was determined as 35 pg µl⁻¹ (signal-to-noise, S/N=3).

Data was analyzed through the non-parametric test of Kruskal-Wallis with SPSS 15.0[©] (SPSS Inc., IL, USA). All values are given as average±S.E.M.

Results

All analyses conducted on *C. nigra* had GABA levels below the LOD. For *P. ferruginea*, the levels were below the LOD for 16/19 samples taken during winter (reproductive season) and 5/14 samples taken during the summer (resting season).

Patella ferruginea pedal mucus contained a significantly higher amount of GABA during the resting season (40.59±14.44 pmol per mg mucus) than during the reproductive period (3.12±1.60 pmol per mg mucus) (*K*=13.63; *P*<0.001). No correlation was found with the size of the individuals (Fig 1a). Mucus production was higher during winter (37.02±5.11 mg mucus per h) than during summer (24.71±5.47 mg mucus per h) (*K*=4.367; *P*<0.05) but showed no correlation with limpet size (Fig 1b).

Discussion

Since the work of Morse et al. (1979) describing the effects of GABA on the settlement of *Haliotis rufescens* larvae, many authors have reported this and other compounds to have larvae-attracting properties in a wide variety of species (see review by Pawlik 1992). For the family Patellidae, only one study by Rivera-Ingraham et al. (2011) experimentally tested the effects of GABA on limpet behavior and recruitment. With the present study we confirm that GABA is naturally secreted in the pedal mucus of *P. ferruginea*, reinforcing the hypothesis that chemical cues, and GABA in particular, play a role in the species life cycle.

Sexually mature *P. ferruginea* can be found in Ceuta from late September to the end of February (personal observation) so settlement should start in January as suggested by Frenkiel (1975). The small size of the new settlers makes it difficult to detect them before they reach a size of about 4 mm, which in natural conditions should take around 2.7 months for animals located inside the port of Ceuta (Espinosa et al. 2008) where our animals were taken. The fact that *P. ferruginea* individuals secrete higher levels of GABA during the reproductive resting

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