Evaluation of non-lethal procedures for gonadal analysis of the West Indian topshell, *Cittarium pica* (Gastropoda: Trochida)

Evaluación de procedimientos no letales para el análisis gonadal del caracol West Indian, *Cittarium pica* (Gastropoda: Trochida)

*Judith Barros **, *Christian Erazo* y *Luz A. Velasco*
Laboratorio de Moluscos y Microalgas, Universidad del Magdalena, Santa Marta Colombia

Abstract

To enable the regular and harmless monitoring of the gonadal development of the threatened tropical marine gastropod *Cittarium pica*, non-lethal procedures for gonadal analysis of adult individuals were evaluated. Ingestion and survival rates were examined in experimental groups of snails that were untreated (controls) and treated with procedures such as shell drilling (D); shell drilling and gonadal biopsy (D+B); shell drilling, gonadal biopsy, and covering the hole in the shell with a soft cover made with soft commercial modeling clay (D+B+SC); shell drilling, gonadal biopsy, and covering the hole of the shell with a hard cover made with acrylic resin (D+B+HC). The different procedures applied did not affect the ingestion rate of the snails, except for those individuals exposed to the D+B+SC treatment, which exhibited a decrease ingestion rate compared to the controls. All treatments caused a decrease in the survival rates of the snails compared to the controls, with shell drilling responsible for a 45 % decrease, and the gonadal biopsy for an additional 39 %. Covering the hole in the shell did not improve these values. The results indicate a high sensitivity of *C. pica* to the manipulation of its shell and tissues, ruling out the use of these procedures for this threatened species

Keys words: gonadal biopsy; shell manipulation; cigua; burgao; whelks

Resumen

Con el fin de viabilizar el monitoreo regular e inocuo del desarrollo gonadal del gasterópodo marino tropical amenazado *Cittarium pica*, se evaluaron procedimientos no letales para el análisis gonadal de individuos adultos. Se examinaron las tasas de ingestión y supervivencia de grupos experimentales de caracoles no tratados (controles) y tratados con procedimientos como perforación de la concha (D); perforación de la concha y biopsia gonadal (D+B); perforación de la concha, biopsia gonadal y obertura del agujero de la concha con una cubierta de plastilina blanda comercial (D+B+SC); perforación de la concha, biopsia gonadal y cobertura del agujero de la concha con una cubierta de resina acrílica dura (D+B+HC). Los diferentes procedimientos aplicados no afectaron la tasa de ingestión de los caracoles, excepto en aquellos individuos expuestos al tratamiento D+B+SC, que mostraron una disminución en la tasa de ingestión en comparación con los controles. Todos los tratamientos causaron una disminución en las tasas de supervivencia de los caracoles en comparación con los controles, siendo la perforación de la concha responsable de una disminución del 45 % y la biopsia gonadal de un 39 % adicional. La cobertura del agujero de la concha no logró mejorar estos valores. Los resultados indican una alta sensibilidad de *C. pica* a la manipulación de su concha y tejidos, descartándose la utilización de estos procedimientos en esta especie amenzada.

Palabras clave: biopsia gonadal; manipulación de la concha; cigua, burgao, whelks

***Autor de correspondencia:** jbarros@unimagdalena.edu.co
Editor: Nancy Yolimar Suárez-Mozo
Recibido: 11 abril de 2025
Aceptado: 23 de mayo de 2025
Publicación en línea: 14 de julio de 2025
Citar como: Barros, J., Erazo, C y Velasco, L. A. (2025). Evaluation of non-lethal procedures for gonadal analysis of the West Indian topshell, *Cittarium pica* (Gastropoda: Trochida). *Intropica*, 20(1) .https://doi.org/10.21676/23897864.6132

INTROPICA

CC BY NC SA

Introduction

Understanding the reproductive biology of a species is crucial to study its life cycle. Generally, reproductive studies use invasive procedures or techniques in which organisms are killed to obtain their reproductive organ (i.e., testis or ovary); however, various non-invasive or semi-invasive techniques have also been proposed and validated as proxy that provides an observation of the reproductive process, while minimizing the impact on natural populations. For instance, non-invasive diagnostic techniques include in vivo magnetic resonance imaging to assess the sexual maturity in the gastropod mollusc *Patella ferruginea* (Guallart *et al.,* 2020), or the use of traditional body ratios and body shape over time to perceive gonad maturity and, in turn, the reproductive season in the polyplacophora mollusc *Chiton articulatus* (Ramirez-Santana *et al.,* 2024). Regarding semi-invasive techniques, these include the gonadal biopsy or germ cell aspiration tested in some mollusk species such as the bivalves *Actinonaias ligamentina*, *Elliptio dilatata* (Saha and Layzer, 2008), *Gomphina veneriformis* (*Lee et al.,* 2010), *Lampsilis straminea* (Beaver *et al.,* 2019), *Quadrula apiculate*, *Quadrula verrucose*, *Quadrula petrina*, *Quadrula houstonensis* (Tzakiris *et al.,* 2016), *Pinctada margaritifera* (Acosta-Salmón and Southgate, 2004), *Tegillaca grano*sa (Lee et al., 2010) and the gastropods *P. ferruginea* (Guallart et al., 2013) and *Pleurobranchaea maculate* (Khor *et al.,* 2013), drilling the shell or cutting a ‘window’ in the shell until the nacre layer and subsequent plugging of hole in the bivalves *Crassostrea virginica* and *Mytilus edulis* (Svärdh, 2003; Yang *et al.,* 2013) as well as in the gastropods *Trochus niloticus* and *Turbo militaris* (Dobson and Lee, 1996; Seinor and Benkendorff, 2023). These non-invasive or semi-invasive techniques suggest little or no signs of stress or death in animals subjected to treatment, thus demonstrating that they are methods with non-lethal results and useful as a proxy of reproduction.

Cittarium pica is a vetigastropod that inhabits the rocky intertidal zone of the Caribbean Sea, which has been used by local coastal communities as a food resource and for handcraft elaboration, to the point of being threatened by overfishing (Chasqui *et al.,* 2022; Daza-Guerra *et al.,* 2018 Osorno *et al*., 2009; Robertson, 2003). *C. pica* exhibits a continuous reproductive cycle, a sexual ratio of 1:1, and the size at sexual maturity between 29 and 68 mm of shell length (Correa *et al.,* 2012; Debrot, 1990; Osorno and Díaz, 2006). It is a dioecious and iteroparous species, with external fertilization, without external sexual dimorphism, with a gonad in apical position, which is dark green color in females and whitish in males (Bell, 1992; Randall, 1964; Velasco and Barros, 2017).

Currently, the production of juveniles C. pica is being developed in hatchery for restocking and aquaculture purposes (Guete-Salazar *et al.,* 2021; Velasco *et al.,* 2019; Velasco and Barros, 2017; 2018). However, its success has been limited by the low spawning and low reproductive conditioning (gonadal ripening or maturation under controlled conditions) responses obtained, mostly due to the difficulties of identifying the sex and monitoring the gonadal development stage of the animals used as broodstock without injuring them (Velasco and Barros, 2017). Our aim was to evaluate the effects of shell drilling, gonadal biopsy, and subsequent covering of the shell hole on the rates of ingestion and survival of *C. pica* adults.

Materials and methods

A total of 450 adult individuals of *C. pica* with sizes higher than those mentioned above at sexual maturity (70.6 ± 10.6 mm in shell length “SL” and 114.4 ± 56.2 g in total live body weight “BW”) were collected in the intertidal zone of Taganga Bay, Colombian Caribbean (11°16'04" N, 74°11'36" W) and then were transported in wet conditions inside isothermal containers to the Laboratory (approximately 15 min walk).

The snails were cleaned of epibionts by abrasion and the flow of micro-filtered (1μm) and UV-sterilized seawater (Velasco and Barros, 2018). The shell of each animal was marked, and its diameter and live weight were measured using a caliper (±0.1 mm) and a scale (±0.001 g) (Velasco and Barros, 2018). Five experimental groups of 90 randomly distributed animals were formed, and each group received a non-lethal procedure as described below (figure 1). D procedure: shell drilling in the apical part (where the gonad is located), using a micro-drill (tip diameter of 1 mm). D+B procedure: shell drilling and gonadal biopsy (around 1 mm of depth and 0.05 mL of gonadal tissue) using a hypodermic syringe (21G x 1 1/2"). D+B+SC procedure: shell drilling, gonadal biopsy, and plugging of shell hole with a soft cover made with soft commercial modeling clay (Kores®). D+B+HC procedure: shell drilling, gonadal biopsy, and plugging of shell hole with a hard cover made with acrylic resin material (Sintesolda®). Control procedure: animals not treated.

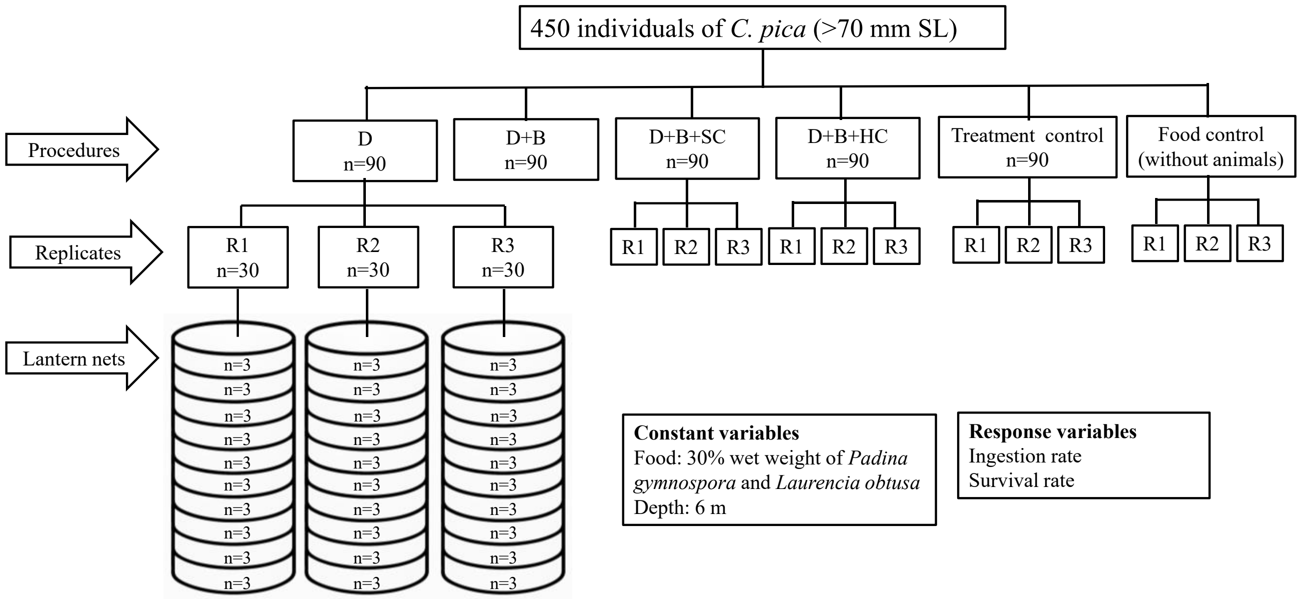

Figure 1. Experimental design used in the present study. Shell drilling (D), gonadal biopsy (B), plugging of shell hole with soft cover (SC), and hard cover (HC).

Later, each experimental group was subdivided into three replica groups of 30 animals randomly assigned. Each replica group was placed in a 10-level lantern-type net (diameter = 45 cm, height = 200 cm, mesh size of 21 mm), evenly distributed on each level (3 animals per level) with one shelter per level (30 cm of a 4" PVC pipe) (Encena *et al.,* 2013; Pereira and Rasse, 2007). Fresh macroalgae of the species Padina gymnospora and Laurencia obtusa were supplied as food, using a 1:1 ratio equivalent to 30 % of the wet weight of live animals (Carreño *et al.,* 2021; Silva-Aciares *et al.,* 2011). The lantern nets were transferred to the sea and suspended from a long line, provided with buoys and an anchoring system, at a depth of 6 m and distanced from each other by a space of 1 m (Velasco *et al.,* 2009). Three additional lanterns without animals, provided with fresh algae, were available to calculate the proportion of food loss not attributable to ingestion by the snails (such as leaching or degradation) (Yusup *et al.,* 2020).

After one week, each lantern net was extracted, and the remaining macroalgae biomass was quantified, as well as the number of live animals (Velasco et al., 2009). The daily ingestion rate of each animal (IR) was estimated from the difference between the wet weight of the algae at the beginning and the end of the experiment, discounting the biomass loss due to leaching or degradation, divided by the number of live snails at the beginning of the trial (modified from Yusup *et al.,* 2020).

IR (g day^{-1} $animal^{-1}$) = (MWi – MWf corr) / (t * Ni)

MWf corr (g) = MWf + MWi - (MWi *LW)

LW = (MWib - MWfb) / MWib

Where MWi = initial macroalgae weight placed in each lantern net, MWf = final macroalgae weight found in each lantern net, MWf corr = final macroalgae weight corrected (adding the biomass loss due to leaching or degradation), LW = fraction of macroalgae weight loss due to leaching or degradation, t = time and Ni= number of live animals in each lantern net at the beginning time.

Survival rate (S) was calculated by quantifying the difference between the number of live animals at the beginning (Ni) and at the end of the experiment (Nf) (Velasco and Barros, 2019).
S (%) = (Ni - Nf) / Ni * 100

To determine the effects of each non-lethal procedure on the rates of ingestion and survival of the snails, data were analyzed using One-Way Analyses of Variance, once the compliance with the assumptions of homoscedasticity (Cochran test) and normality (Shapiro Wilks analysis) of the response variables was confirmed (Zar, 1999). Finally, Spearman correlation analysis was performed between the rates of ingestion and survival of the snails. The analyses were performed using the statistical software Statgraphics Centurion XVI.I and statistical significance was accepted at $P < 0.05$.

Results

The ingestion rates of *C. pica* adults ranged from 1.9 to 2.9 g day^{-1} animal^{-1} (figura. 2A). Lower values were exhibited by the group treated with D+B+SC compared to the control group (df = 4, F = 6.49, p = 0.0077). Regarding the other three treatments (D, D+B and D+B+HC), they did not show statistically significant differences compared to the control ($p > 0.05$).

Survival rates ranged from 4 to 97 % (figure 2B), with the highest values found in the control group, followed by those groups with animals whose shells were drilled, and the lowest values found in the groups with animals in which shell drilling and subsequent gonadal biopsy were performed, regardless of whether or not the shell hole was later covered (df = 4, F = 73.51, $p < 0.0001$).

A significant positive correlation was found between the rates of ingestion and survival (r = 0.6882, n = 15, p = 0.0100).

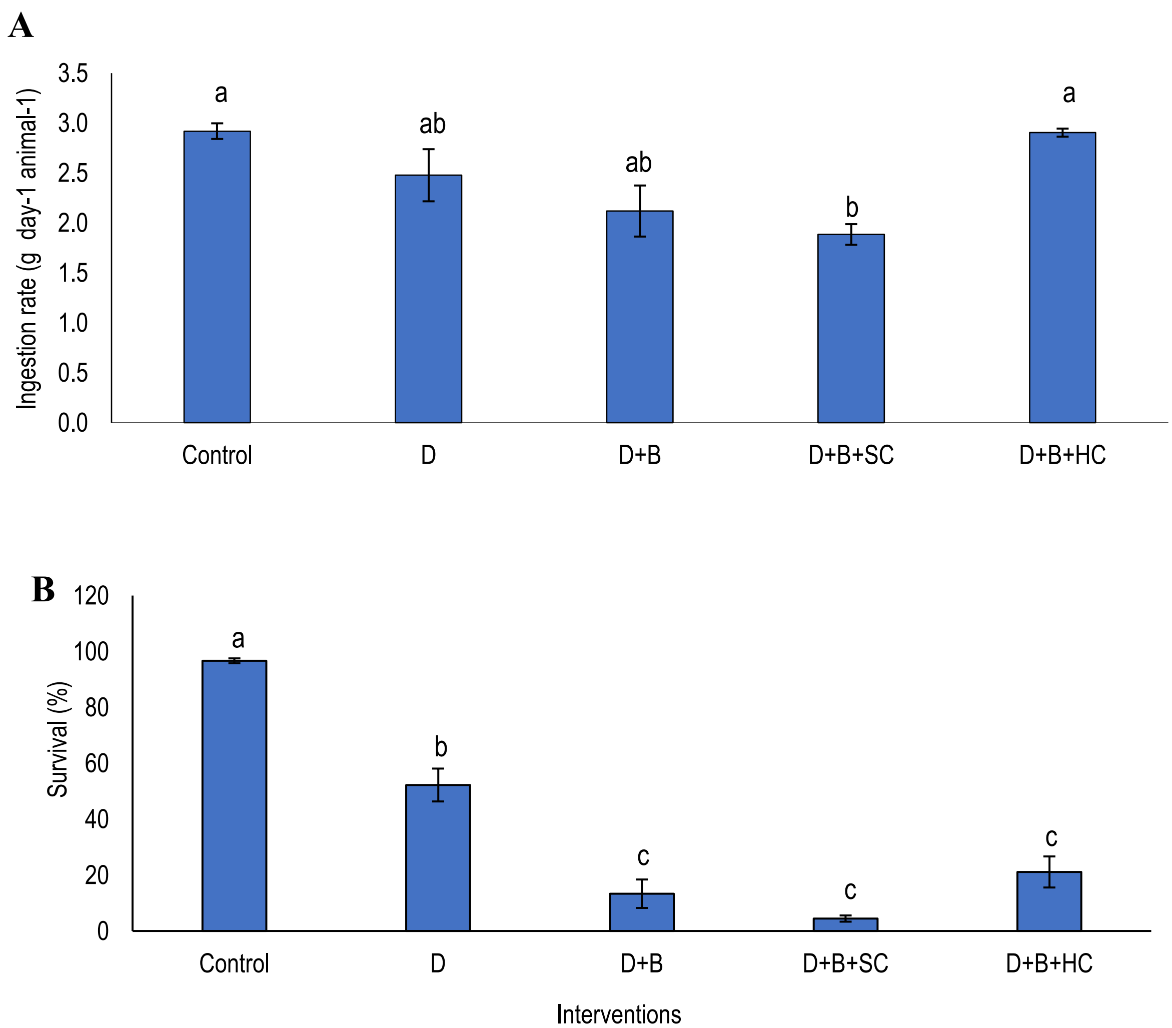

Figure. 2. Effects of each non-lethal procedure in the ingestion (A) and survival (B) rates of *Citarium pica* adults. Abbreviations to treatments: Shell drilling (D), gonadal biopsy (B), plugging of shell hole with soft cover (SC), and hard cover (HC), and not treated (control). Values are means ± Standard Error. Different letters indicate significant differences among treatments at $p < 0.05$.

Discussion

The similarity of the ingestion rates for most of the snails treated and untreated (control animals), suggests that those interventions did not significantly impact the ingestion capability, consistent with findings for *T. niloticus* subjected to shell drilling (Dobson and Lee, 1996). However, the lower ingestion rates observed in the snails exposed to the procedures of shell drilling, gonadal biopsy and covering of the shell hole with plasticine (D+B+SC), can be attributed to the interference of this soft material with their grazing activity and to the lower survival rate in this procedure at the end of the experiment.

The reduction of 45 % in the survival rates of *C. pica* snails subjected to shell drilling contradicts the lack of effect of this procedure registered in *C. virginica*, *M. edulis*, *T. niloticus* and *Turbo militaris* (Dobson and Lee, 1996; Seinor and Benkendorff, 2021; Svärdh, 2003; Yang *et al.,* 2013). Such results suggest that the drilling process may cause stress or small tissue damage, not macroscopically noticeable. The additional decrease in the survival rates of *C. pica* treated with gonadal biopsy (of around 39 %) is similar to the results observed in *C. virginica* (Yang *et al.,* 2013), but it contradicts the lack of effect of this procedure found in *M. edulis* and *T. militaris* (Seinor and Benkendorff, 2021; Svärdh, 2003). Such results indicate that the damage of the gonadal tissue caused by the hypodermic needle in *C. pica* exacerbated the negative effect caused by the shell drilling. The lack of effect of the shell hole sealing, with either a soft or a hard material, on the survival rate of *C. pica* contradicts the negative effects of this procedure registered in *M. edulis* and *T. militaris* (Seinor and Benkendorff, 2021; Svärdh, 2003). Therefore, the physical protection of the treated gonadic tissue from the action of external microorganisms did not seem important for the survival improving of *C. pica* treated.

The survival rates of *C. pica* adults subjected to shell drilling and/or gonadal biopsy (4-52 %) were relatively low or similar to values reported for other mollusk such as *C. virginica* (80-100 %; Yang *et al.,* 2013), M. edulis (5-88 %; Svärdh, 2003), *T. niloticus* and *T. militaris* (100 %; Dobson and Lee, 1996; Seinor and Benkendorff, 2021). The negative results obtained in this study suggest a high sensitivity of *C. pica* to the shell drilling and gonadal damage, being necessary to improve the surgical instruments, drilling and sampling techniques as well as the aseptic measures to avoid infections (Berzins and Smolowitz, 2006). In addition, the use of anesthesia can reduce stress during the intervention and improve the survival rate (Acosta-Salmón and Southgate, 2004). Currently, the spawning of *C. pica* under hatchery conditions during daytime and coinciding with new moon, flooding and high tides (Velasco and Barros, 2017) remains the only viable non-destructive technique to differentiate sex and identify mature animals in this species.

Conclusions

Although shell drilling and gonadal biopsy did not seem to result in a noticeable reduction in the ingestion rate of C. pica, they significantly decreased their survival rate (between 48 and 96 %), while covering the shell hole did not bring about any improvement in the survival rate. Therefore, this species is sensitive to any alteration in shell integrity and soft tissues, rendering these procedures unsuitable, especially considering the vulnerable conservation status of its natural populations.

Ethical declarations

The handling of experimental animals is endorsed by the Research Ethics Committee of the University of Magdalena, in compliance with the provisions of Rectoral Resolution 427 of 2018 and in accordance with the ethical principles of autonomy and justice.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Judith Barros and Christian Erazo: performed the experiments, analyzed the data and wrote this manuscript.

Luz A. Velasco: provided the original idea, advised the experiments, and wrote this manuscript.

Acknowledgements

The authors would like to thank the staff of the Marine Mollusk Group of the University of Magdalena. This research was conducted with the permits from the Regional Autonomous Corporation of Magdalena (CORPAMAG) 761 of 05/18/2010, 670 of 03/28/2012 and 366 of 02/23/2017. It was supported by the projects COLCIENCIAS 1117-521-28882 and FONCIENCIAS 022-2016 and 0025-2019.

References

Acosta-Salmón, H. and Southgate, P.C. (2004). Use of a biopsy technique to obtain gonad tissue from the blacklip pearl oyster *Pinctada margaritifera* (L.). *Aquaculture Research*, 35, 93-96. https://doi.org/10.1111/j.1365-2109.2004.00988.x

Beaver, C.E., Geda, S.R., and Johnson, N.A. (2019). Standardizing a non-lethal method for characterizing the reproductive status and larval development of freshwater mussels (Bivalvia: Unionida). Journal of Visualized Experiments, 152, e60244. https://doi.org/10.3791/60244

Bell, L. (1992). Reproduction and larval development of the West Indian topshell, *Cittarium pica* (Trochidae) in the Bahamas. *Bulletin Marine Science*, 51(2), 250-266.

Berzins, I.K., and Smolowitz, R. (2006). Diagnostic Techniques and Sample Handling. In: G.A. Lewbart (Ed.). *Invertebrate Medicine* (pp 263-274), Blackwell Publishing Ltd. Oxford.

Carreño, A., Velasco, L.A., and Toro, B. (2021). Effect of diet and water quality on the energetic physiology of the West Indian top shell *Cittarium pica*. *Aquaculture*, 531, 735889. https://doi.org/10.1016/j.aquaculture.2020.735889

Chasqui, V.L., Alvarado-Chacón, E.M., Ardila, N., Borrero-Pérez, G.H., Campos N.H., and Mejía-Quintero, K. (Eds.). (2022). *Libro rojo de invertebrados marinos de Colombia*. Instituto de Investigaciones Marinas y Costeras INVEMAR, Ministerio de Ambiente y Desarrollo Sostenible. Serie de publicaciones generales de INVEMAR No. 122. Santa Marta, Colombia. 388. ISBN digital: 978-958-8935-76-8.

Correa, T., Toro, B., and Rosique, J. (2012). Some aspects of the bioecology of the west indian topshell *Cittarium pica* (Mollusca : Gastropoda) in the Darien Colombian Caribbean. *Boletín Científico Museo de Historia Natural*, 16, 162-172.

Daza-Guerra, C.A., Martínez-Hernández, N.J., and Narváez-Barandica, J.C. (2018). Population aspects of burgao *Cittarium pica* (Gastropoda: Tegulidae) in the rocky shore of Santa Marta, Magdalena, Colombia. *Revista Mexicana de Biodiversidad*, 89, 430-442. https://doi.org/10.22201/ib.20078706e.2018.1.2356

Debrot, A. (1990). Survival, growth and fecundity of the west indian topshell, *Cittarium pica* (Linnaeus), in various rocky intertidal habitats of the Exuma Cays, Bahamas. *The Veliger*, 33, 363-371.

Dobson, G., and Lee, C.L. (1996). Improved method of determining the sex of the marine topshell, *Trochus niloticus* (Mollusca: Gastropoda) for spawning. *Aquaculture*, 139, 329-331.

Encena, V.C., De La Pena, M., and Balinas, V.T. (2013). Evaluation of density and cage design for the nursery and grow-out of the tropical abalone *Haliotis asinina* Linne 1758. *Journal of Shellfish Research*, 32(1), 21–27.

Guallart, J., Ferranti, M.P., Bacigalupo, L., and Chiantore, M. (2020). In vivo magnetic resonance imaging to assess the sexual maturity of the endangered limpet *Patella ferruginea*. *Journal of Molluscan Studies*, 86, 422–426. https://doi.org/10.1093/mollus/eyaa021

Guete-Salazar, C., Barros, J., and Velasco, L.A. (2021). Settlement and culture of post-larvae, spat and juveniles of the West Indian topshell, *Cittarium pica* (Linne, 1758). *Aquaculture*, 543, 737029. https://doi.org/10.1016/j.aquaculture.2021.737029

Khor, S., Wood, S.A., Salvitti, L., Ragg, N.L.C., Taylor, D., McNabb, P., and Cary, S.C. (2013). Development of a non-lethal biopsy technique for estimating total tetrodotoxin concentrations in the grey side-gilled sea slug *Pleurobranchaea maculata*. *Toxicon*, 74, 27–33. https://doi.org/10.1016/j.toxicon.2013.07.012

Lee, J.S., Ju, S.M., Park. J.S., Park, Y.G., Shin, Y.K., and Park, J.J. (2010). Germ cell aspiration (GCA) method as a non-fatal technique for sex identification in two bivalves (*Gomphina veneriformis* and *Tegillaca granosa*). *Development and Reproduction*, 14(1), 7-11.

Pereira, L., and Rasse, S. (2007). Evaluation of growth and survival of juveniles of the Japanese abalone *Haliotis discus hannai* in two culture systems suspended in tanks. *Journal of Shellfish Research*, 26(3), 769–776. https://doi.org/10.2983/0730-8000(2007)26[769:EOGASO]2.0.CO;2

Osorno, A., and Diaz, J.M. (2006). Explotación, usos y estado actual de la cigua o burgao *Cittarium pica* (Mollusca: Gastropoda: Trochidae) en la costa continental del Caribe colombiano. *Boletín de Investigaciones Marinas y Costeras*, 35, 133-148.

Osorno, A., Gil, D.L. and Gómez, L.A. (2009). *Plan de investigación para la conservación de Cittarium pica* (L. 1758). Invemar, Serie de publicaciones especiales 16.

Ramirez-Santana, B.P., Alvarez-Garcia, I.L., Avila-Poveda, O.H., Arellano-Martinez, M., and Ospina-Garces, S.M. (2024). Seasonal dimorphism as an expression of sexual dimorphism: Influence of gonad maturity on the body shape of a rocky intertidal polyplacophoran. *Zoology*, 167: e126224. https://doi.org/10.1016/j.zool.2024.126224Randall, H.E. (1964). A study of the growth and other aspects of the biology of the West Indian Top Shell *Cittarium pica* (Linnaeus). Bulletin Marine Science, 14, 424-443.

Robertson, R. (2003). The edible West Indian Whelk Cittarium pica (Gastropoda: Trochidae): natural history with new observations. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 153, 27-47.

Saha, S., and Layzer, J.B. (2008). Evaluation of a nonlethal technique for determining sex of freshwater mussels. *Journal of the North American Benthological Society*, 27(1), 84-89.

Seinor, K. and Benkendorff, K. (2023). Shell drilling and gonad biopsies provide a rapid, nonlethal method for in situ assessment of reproductive periodicity in a turbinid gastropod. *Journal of Molluscan Studies*, 89, eyad012. https://doi.org/10.1093/mollus/eyad012.

Silva-Aciares, F.R., Carvajal, P.O., Mejías, C.A., and Riquelme, C.E. (2011). Use of macroalgae supplemented with probiotics in the Haliotis rufescens (Swainson, 1822) culture in Northern Chile. *Aquaculture Research*, 42, 953-961.

Svärdh, L. (2003). Tissue sampling from live blue mussels, Mytilus edulis, a field study from the Swedish west coast. *Journal of Sea Research*, 49, 221-225.

Tzakiris, E.T., Randklev, C.R., and Conway, K.W. (2016). Effectiveness of a nonlethal method to quantify gamete production in freshwater mussels. *Freshwater Science*, 35(3), 958–973.

Velasco, L.A., and Barros, J. (2017). Spawning and early development of the West Indian top shell, *Cittarium pica* (Linnaeus, 1758), under ex-situ conditions. *Aquatic Living Resources*, 30, 22. https://doi.org/10.1051/alr/2017022

Velasco, L.A., and Barros, J. (2018). Experimental culture of larvae, post-larvae and juveniles of the West Indian top shell, *Cittarium pica* (Linnaeus, 1758). *Aquaculture Research*, 49, 1628-1637. https://doi.org/10.1111/are.13618

Velasco, L.A., and Barros, J. (2019). Survival and growth of the *Caribbean scallops*, *Argopecten nucleus* and *Nodipecten nodosus*, in suspended systems at different culture depths and net replacement frequencies. *Aquaculture Environment Interactions*, 11: 537–549. https://doi.org/10.3354/aei00334

Velasco, L.A., Barros, J., Guerrero, A., 2009. Growth and survival of the Caribbean scallops *Argopecten nucleus* and *Nodipecten nodosus* in suspended culture at the Taganga Bay, Colombia. *Aquaculture Research*, 40, 687–695.

Velasco, L.A., Villarruel, Y., and Toro, B. (2019). Nursery culture of wild and hatchery-produced juveniles of the West Indian top shell, *Cittarium pica*, fed microalgal and artificial biofilms. *Aquaculture International*, 27, 1289–1299. https://doi.org/10.1007/s10499-019-00386-2

Yang, H., Supan, J., Guo, X., and Tiersch, T.R. (2013). Nonlethal sperm collection and cryopreservation in the Eastern Oyster Crassostrea virginica. *Journal Shellfish Research*, 32, 429-437. https://doi.org/10.2983/035.032.0217

Yusup, D.S., Mahardika, I.G., Suarna, I.W., and Giri, I.N.A. (2020). Feeding preference and growth response of early adults abalone, *Haliotis squamata* on some macroalgae. *Biodiversitas*, 21(9), 4369-4375. https://doi.org/10.13057/biodiv/d210950

Zar, J.H., (1999). *Biostatistical analysis*. Pearson Education India.