Asimetría fluctuante en *Hypolobocera bouvieri* (*Decapoda:Pseudothelphusidae*) en microcuencas andinas de Colombia bajo distintos grados de intervención humana

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Undergraduate Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Bachelor of Biology

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Dedication

Un apoyo incondicional en la formación que he tenido desde mi infancia hasta mi presente, sin duda alguna ha sido mi abuelo, la música, el arte, la ciencia y las humanidades; fueron algunas de las pocas cosas que me enseñó de forma directa o indirecta, su afán por querer que leyera, que no viera televisión, de alguna forma inspiró mi pequeña mente en búsqueda de nuevas experiencias, por eso, mi abuelito, quisiera dedicarte este triunfo a ti, a ti que a veces no me recuerdas, a ti que a veces olvidas quien eres, a ti quien desearía poder recordar inmortalizado en cada letra de este escrito, a ti, y a todo lo que fuiste.

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Abstract

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Key words: Fluctuating asymmetry, freshwater crabs, anthropogenic disturbance, geometric morphometrics.

Description: Developmental stability is a fundamental biological property that reflects an organism's ability to produce a consistent phenotype under certain environmental conditions. Fluctuating asymmetry (FA) consists of small, random deviations from perfect bilateral symmetry and has been widely used as a sensitive indicator of environmental stress. Despite this, studies addressing FA in Neotropical freshwater crabs remain scarce, particularly within the Pseudothelphusidae family. This study represents the first assessment of fluctuating asymmetry in *Hypolobocera bouvieri* and the first study conducted on pseudothelphusid crabs in Latin America. We aimed to analyze FA in *H. bouvieri* using geometric morphometrics techniques across Andean micro-watersheds with different levels of anthropogenic disturbance in Santander, Colombia, and to evaluate its relationship with physicochemical and environmental variables. A total of 50 individuals were collected from five municipalities. Dorsal carapace images were digitized using 19 landmarks and analyzed using Procrustes ANOVA, PCA, discriminant function analysis and GLMM. Results revealed that fluctuating asymmetry was present among the populations, as well as significant differences in symmetric components of the shape. PCA of the symmetric component revealed two major morphological clusters, mainly separating Santa Bárbara and part of Rionegro from the other municipalities. However, no significant relationship was found between asymmetry scores and measured environmental variables or Habitat Integrity Index (HII) values. These findings suggest that although carapace morphology varies geographically, this variation may not be directly explained by local physicochemical conditions. The broad ecological tolerance and wide distribution of *H. bouvieri* across different disturbed environments could explain the limited variation in fluctuating asymmetry observed among populations.

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Resumen

Título: Asimetría fluctuante en *Hypolobocera bouvieri* (Decapoda:Pseudothelphusidae) en microcuencas andinas de Colombia bajo distintos grados de intervención humana*

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Palabras clave: Asimetría fluctuante, cangrejos dulceacuícolas, perturbación antrópica, morfometría geométrica.

Descripción: La estabilidad del desarrollo es una propiedad biológica que refleja la capacidad de un organismo para producir un fenotipo consistente bajo determinadas condiciones ambientales. La asimetría fluctuante (AF), definida como pequeñas desviaciones aleatorias de la simetría bilateral, es un indicador ampliamente utilizado del estrés ambiental. Sin embargo, los estudios sobre AF en cangrejos dulceacuícolas neotropicales son escasos, especialmente en la familia Pseudothelphusidae. Este estudio constituye la primera evaluación de la AF en Hypolobocera bouvieri y el primer trabajo realizado en pseudotelfúsidos de América Latina. Se analizó la AF mediante morfometría geométrica en cinco microcuencas andinas de Santander, Colombia, con distintos niveles de intervención antrópica. Se recolectaron 50 individuos y se digitalizaron 19 landmarks del caparazón dorsal. Los datos se analizaron mediante ANOVA de Procrustes, análisis de componentes principales (PCA), análisis de función discriminante y modelos lineales generalizados mixtos (GLMM). Los resultados evidenciaron la presencia de AF y diferencias significativas en el componente simétrico de la forma. El PCA mostró dos grupos morfológicos principales, y no se encontró relación significativa entre la asimetría fluctuante y las variables ambientales. Estos resultados sugieren que la variación morfológica de H. bouvieri no está explicada por las condiciones ambientales locales y que su amplia tolerancia ecológica podría explicar la escasa variación en asimetría fluctuante entre los sitios.

* Trabajo de Grado

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Introduction

Developmental stability is a fundamental biological property defined as the ability of an organism to produce a consistent phenotype under a given set of environmental conditions (Benítez et al., 2020; Palmer, 1986, 1994, 1996). However, stress factors acting during ontogeny may alter this process, resulting in fluctuating asymmetry (FA) (H. Benítez et al., 2020b; H. A. Benítez & Parra, 2011; Klingenberg, 2015); small random deviations from perfect bilateral symmetry that arise during development (H. A. Benítez & Parra, 2011; Palmer, 1994). Therefore, fluctuating asymmetry has been widely used in ecology across diverse animal groups as a sensitive indicator of environmental stress (Allenbach, 2011; Arundell et al., 2019; Gabriel N. Castillo & Cynthia J. González-Rivas, 2023; Lens et al., 1999), allowing to assess organismal responses to various ecological disturbances and habitat modification, such as anthropogenic intervention (Frota et al., 2019; Ottaviano & Scapini, 2010; Vilisics et al., 2005).

One of these groups are the crustaceans, for which numerous studies have evaluated their responses to habitat alteration in both marine and freshwater ecosystems. These investigations have included a wide variety of organisms, such as amphipods, cirripeds, isopods, and the group of particular interest in this study, the brachyurans (Duarte et al., 2008; Vilisics et al., 2005; Sullivan, 1998). Brachyurans, or true crabs, constitute one of the best-known groups of crustaceans and have been the subject of extensive research, including studies focused on fluctuating asymmetry (Grinang et al., 2019; Fuller et al., 2022; Fray, 1992). Their biological characteristics allow them to occupy a broad range of habitats, including coastal, marine, brackish, freshwater, and even mountainous environments, the latter being the focus of the present study. Despite receiving less public attention, mountain crabs exhibit a broad geographic distribution, occurring throughout Asia from Japan to Indonesia and throughout the Americas from Mexico to Bolivia (Acevedo-Alonso & Cumberlidge, 2022). Among these mountain crabs are the pseudothelphusid crabs, a family of Neotropical freshwater crabs characterized by high levels of endemism, particularly in Colombia (Acevedo-Alonso & Cumberlidge, 2022) and a pseudo-lung, a modified branchial chamber that allows them to breath air for extensive periods of time (Díaz & Rodríguez, 1977).

The Pseudothelphusidae family constitutes an important component of neotropical aquatic ecosystems, actively participating in processes such as organic matter recycling and trophic dynamics (Acevedo-Alonso & Cumberlidge, 2022; Campos, 2023; Campos et al., 2018). Within this family, *Hypolobocera bouvieri* is the focal species of this study. Its wide distribution across Colombian Andean ecosystems and its occurrence in habitats subjected to different levels of anthropogenic disturbance make it an excellent model for evaluating fluctuating asymmetry (Campos, 2003, 2005, 2015). Despite these characteristics, studies aimed at evaluating its morphological variation or ecology remain limited, particularly those employing geometric morphometric approaches.

In Colombia, studies addressing fluctuating asymmetry have been conducted mainly in vertebrates and insects (Allenbach, 2011; Lens & Van Dongen, 2000; Gastaldo et al., 2024; Lens et al., 1999; Lens & Van Dongen, 2000), while its application in freshwater mountain crabs is virtually nonexistent. This knowledge gap is particularly important to address, Colombia ranks as the second most diverse country in the world for freshwater crabs, surpassed only by China, and approximately 86% of its species are endemic (Acevedo-Alonso & Cumberlidge, 2022). Approximately 25% of the species within the family Pseudothelphusidae are currently classified under some category of conservation concern, primarily as a result of anthropogenic impacts on freshwater ecosystems (Campos & Lasso, 2015). Furthermore, the use of freshwater crabs as ecological proxies may provide valuable insights into the responses of other macroinvertebrate groups to environmental disturbance, allowing broader inferences to be made regarding the effects of human activities on freshwater communities.

Consequently, there is a pressing need to promote studies that not only describe species diversity but also investigate their ecology, particularly the effects of increasing human activities on their habitats and the responses of these organisms to environmental change. To address this gap, the present study constitutes the first analysis of fluctuating asymmetry performed on *Hypolobocera bouvieri* in the country and the first in Latin America for pseudothelphusid crabs, providing a novel contribution to the understanding of the relationship between environmental stress and morphological variation in these organisms. This research aimed to analyze fluctuating asymmetry in *H. bouvieri* through the application of geometric morphometric techniques in Andean micro-watersheds with different levels of anthropogenic disturbance in the department of

Santander (Colombia), and to evaluate the relationship between fluctuating asymmetry, physicochemical variables, and environmental conditions.

1. Objectives

1.1 General Objective

To assess the effects of anthropogenic disturbance on fluctuating asymmetry in populations of *Hypolobocera bouvieri* from Andean micro-watersheds and determine its association with physicochemical and environmental conditions.

1.2 Specific Objectives

To characterize the environmental conditions of the micro-watersheds inhabited by *Hypolobocera bouvieri* based on physicochemical variables and indicators of anthropogenic disturbance.

To quantify fluctuating asymmetry in *H. bouvieri* using geometric morphometric techniques.

To evaluate the relationship between fluctuating asymmetry, physicochemical variables, and the level of anthropogenic disturbance across Andean micro-watersheds.

2. Main Text

2.1 Referential frame

Theoretical and Conceptual Framework

Fluctuating asymmetry (FA), defined as small and random deviations from perfect bilateral symmetry, has long been used as an indicator of developmental instability in natural populations. The concept was formally developed by Palmer (1986), who established the methodological and statistical foundations for its study. Since then, FA has been widely applied to assess the effects of environmental stress, with numerous studies reporting increased asymmetry in organisms exposed to factors such as chemical pollution, habitat fragmentation, and ecosystem degradation (Borgwardt et al., 2019; Dodds et al., 2013; Lens & Van Dongen, 2000). However, the relationship between FA and environmental stress remains debated, as asymmetry patterns may also be shaped by population-specific processes and methodological limitations, complicating their interpretation. Advances in geometric morphometrics have improved the study of FA by providing a robust framework for quantifying morphological variation. Unlike traditional morphometric approaches based on linear measurements, geometric morphometrics uses homologous landmarks to capture the geometry of structures and analyze subtle shape differences with greater precision. This approach is particularly valuable for FA studies because it enables the detection of complex patterns of shape variation while minimizing the biases associated with conventional measurements (H. A. Benítez & Püschel, 2014; Klingenberg, 2015).

Fluctuating asymmetry has been used in many animal groups (Allenbach, 2011; Gabriel N. Castillo & Cynthia J. González-Rivas, 2023; Gastaldo et al., 2024; Lens et al., 1999; Lens & Van Dongen, 2000) including different crustacean groups (Grinang et al., 2019; Arundell et al., 2019; Duarte et al., 2008; Vilisics et al., 2005; Sullivan, 1998, Tobo et al., 2012, Ottaviano & Scapini, 2010), but only a few focused on assessing the stress caused by pollution and habitat degradation by human action (Frota et al., 2019). However, its application in neotropical freshwater mountain crabs is nonexistent, despite the similarities with some other crustacean groups, like the *Orconectes* crayfish, their response to habitat changes may differ, for example, in the study made by Fray (1992) they found that higher concentrations of heavy metals dissolved in water doesn’t correlate with the fluctuating asymmetry, due to some physiological mechanisms on the species that allow them to resist the contaminants. Therefore, the limited understanding of how pseudothelphusid crabs respond to disturbance gradients, their well-known conservation vulnerability and high degree of endemism highlights the need for further investigation to better understand their ecological and environmental dynamics.

State of the Art

Research on fluctuating asymmetry have reported variable patterns among among taxa, with some showing robust correlations with environmental disturbance and others exhibiting inconsistent responses (Allenbach, 2011; Clarke, 1993; Lens & Van Dongen, 2000, 2000; Nishizaki et al., 2015). In Colombia, fluctuating asymmetry research has focused mainly on fish, insects, and reptiles (Allenbach, 2011; De La Torre et al., 2007; Gabriel N. Castillo & Cynthia J. González-Rivas, 2023; Gastaldo et al., 2024b; Lens et al., 1999; Lens & Van Dongen, 2000) Although crustaceans, and particularly freshwater mountain crabs, remain underrepresented in fluctuating asymmetry research, studies conducted among other animal groups have revealed contrasting patterns that provide useful context for interpreting potential responses in local species. Investigations on coastal crabs have reported morphological responses associated with habitat alteration (Frota et al., 2019), whereas studies on freshwater crabs have suggested that fluctuating asymmetry is not always directly linked to environmental stress and may instead reflect ecological or functional traits of the species (Tobo et al., 2012; Grinang et al., 2019). Furthermore, research on crayfish has shown that some species may buffer the effects of contaminants through physiological mechanisms, resulting in limited or undetectable impacts on developmental stability (Tobo et al., 2012; Fray, 1992). These contrasting findings highlight the complexity of crustacean responses to environmental stress and reinforce the importance of evaluating such patterns in Neotropical freshwater mountain crabs.

Research on freshwater mountain crabs, particularly within the family Pseudothelphusidae, has focused primarily on taxonomic and systematic perspectives rather than ecological ones, leaving largely unexplored the ways in which these organisms respond to habitat alteration and environmental change. Although some ecological studies have been conducted (Campos, 2005, 2015; Rivera-Pérez, 2023), significant gaps remain in our understanding of their ecological responses to anthropogenic pressures. Addressing this aspect is particularly important given that Colombia harbors the second-highest diversity of freshwater crabs worldwide, with approximately 86% of its species being endemic and with approximately a quarter of its species classified within a threatened category. This remarkable diversity and threat status highlights the need for research that not only documents species diversity but also investigates the interactions between freshwater mountain crabs and human-induced environmental impacts, such studies are essential for improving our understanding of their ecology and for supporting future conservation efforts.

Legal Framework

Specimen euthanasia was conducted in accordance with Law 1774 of 2016 of the Congress of the Republic of Colombia, Law 84 of 1989 of the National Animal Protection Statute, and Law 576 of 2000 of the Veterinary Code of Ethics.

Specimen collection was carried out under Resolution 0047 of January 2015 issued by ANLA (National Environmental Licensing Authority), Articles 8, 27, and 69 of the Political Constitution of Colombia, Decree 644 of March 23, 1999, the collection permit for national research institutions under Article 7 requiring compliance with the obligations established in Article 90 of Decree 1376 of 2013 issued by ANLA, and Resolution 001220 of April 24, 2026.

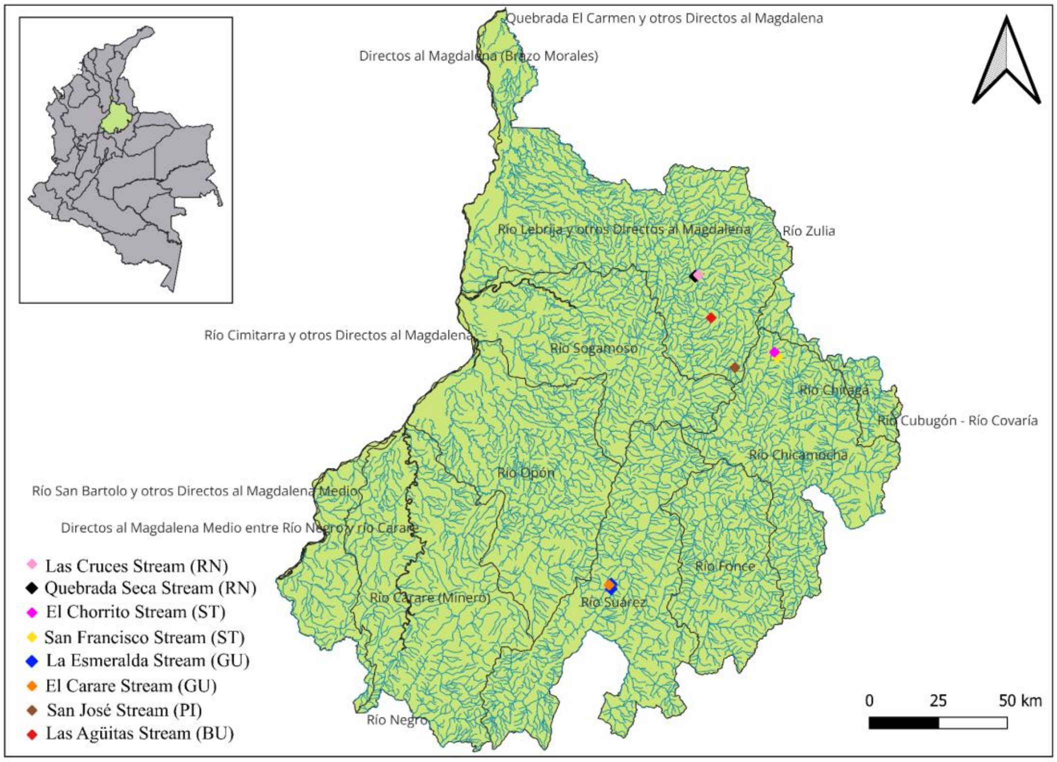
2.1.1 Methods.

Study Area

This project was conducted in five municipalities in the department of Santander (Colombia). Within each municipality, two localities were selected based on previous records of *H. bouvieri* and information provided by the local community. These municipalities were: Bucaramanga (Comuna Morrorico, Miraflores neighborhood, with two sampling sites in Quebrada Las Agüitas, 7.1285604N -73.10365283W); Piedecuesta (Vereda San José, with two sampling sites in Quebrada San José, 6.964817167N -73.02656862W); Rionegro (Quebrada Seca locality, with one sampling site 7.264273333N -73.15546W; Quebrada Las Cruces locality, with one sampling site 7.268803333N -73.14533667W); Guadalupe (Vereda El Carare locality, with two sampling sites in Quebrada El Carare, 6.24000315N -73.43063597W); and Santa Bárbara (Vereda

San Francisco locality with one sampling site in Quebrada San Francisco, 6.998165N -72.88739333W; Vereda Esparta locality with one sampling site in Quebrada El Chorrito, 7.003028333N -72.88626167W). Elevation across the study area ranges from 700 masl to 2400 masl, with Rionegro being the lowest and Santa Bárbara the highest. (Figure 1)

Figure 1 – Map of Santander (Colombia - South America) with the municipality sites



Note: Rionegro (RN); Santa Bárbara (ST); Guadalupe (GU); Bucaramanga (BU); Piedecuesta (PI).

Specimen Collection, Field Data Record and Site Characterization

Specimens were collected following the methodology of Peter K.L. (2016), searching beneath rocks ranging between 30 cm and 70 cm in small streams with firm rocky substrates, abundant leaf litter, and dense vegetation cover. Collections were conducted between November 2025 and March 2026 during the dry season, between 7:00 AM and 12:00 PM. Individuals were euthanized through thermal shock using a container filled with ice and cold water, also following Peter K.L. (2016), and subsequently preserved in 70% ethanol. At each specimen encounter and collection event, physicochemical variables were recorded using an APERA multiparameter probe, including pH, electrical conductivity (EC), total dissolved solids (TDS), and water temperature (Temp).

Additionally, habitat quality was assessed using the Habitat Integrity Index (HII) (Nessimian et al., 2008), which integrates 12 geomorphological and habitat attributes, including sediment composition, vegetation cover, and channel morphology. HII scores, together with physicochemical variables, were used as proxies of anthropogenic disturbance because human activities influence both habitat structure and water quality. A Principal Component Analysis (PCA) was conducted to summarize environmental variation and characterize disturbance gradients among sampling sites (Angeler et al., 2014; Borgwardt et al., 2019; Dodds et al., 2013; Friberg, 2014; Gwynfryn Jones, 2001; O'Brien et al., 2016).

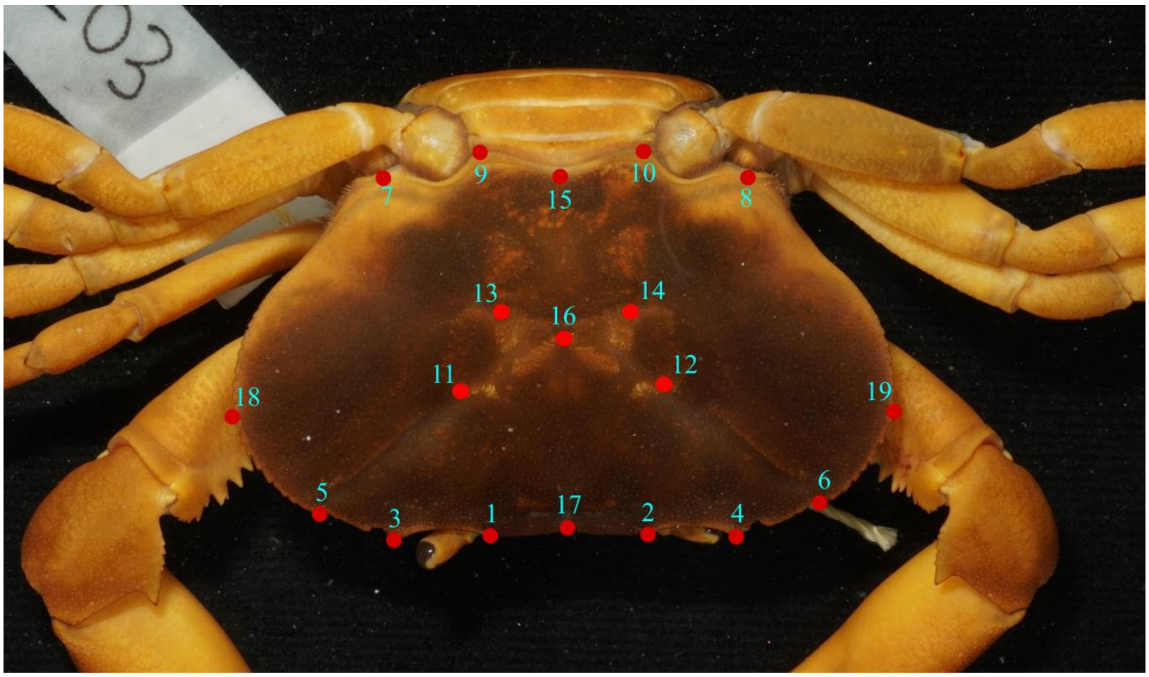
Photography and Digitization

Specimen digitization was carried out through dorsal-view carapace photography. Images were taken using a standardized setup designed to minimize angular distortion. This consisted of a Sony Alpha 6400 camera mounted on a tripod with the lens directed downward. The specimen was placed beneath it, submerged in water inside a transparent plastic container with a black background, held in place with needles, and accompanied by a ruler for scale calibration. A label containing specimen information (collection site, locality, sex, and individual number within the site) was included in each image. Image digitization was performed using Imaging Edge version 1.2.02 (Sony Corporation, 2026). Images were sorted into folders according to the collection site and converted to TIFF format.

The TIFF images were processed in tpsUtil32 (Rohlf, 2023), where they were merged into a single TPS file while preserving the specimen nomenclature. To capture carapace shape variation, 19 landmarks were digitized using tpsDig2 version 2.32 (Rohlf, 2021) at carapace edges, projections, and branchial chamber regions.

Since this is the first study of this type in Pseudothelphusidae, no prior landmark configuration existed beyond Campos (1986). Therefore we aimed to characterize variation in the dorsal region of the carapace by capturing a series of homologous anatomical structures that could be consistently identified across individuals, including margins, projections, and visible features of the branchial chamber. However, some other freshwater mountain crabs have a similar landmark configuration (Grinang et al., 2019) as the one proposed in Figure 2. Then, landmark digitization was repeated on two separate days to generate two TPS files representing digitization error.

Figure 2 – Landmarks displayed on the dorsal surface of the carapace.



Data Analysis

Following the methods made by Benítez & Püschel (2014), the two TPS files were imported and analyzed in MorphoJ version 1.08 (Klingenberg, 2011). They were merged into a single dataset and classified according to metadata recorded in the photographic nomenclature (site, sex, and individual identity). To eliminate effects of size, position, and orientation, a Procrustes superimposition was performed. This was followed by a Procrustes ANOVA incorporating site effects to obtain the individual mahalanobis asymmetry values, which are a numeric value that resumes the FA magnitude for each individual, and differences in landmark centroid configurations. These asymmetry values were extracted and stored in an additional .txt file. Using mahalanobis asymmetry values and the symmetric shape component, a regression analysis was conducted to examine if a certain shape presents particularly levels of fluctuating asymmetry.

Two covariance matrices were subsequently constructed from the combined dataset: one representing the symmetric component of shape variation and the other representing the asymmetric component. The symmetric component describes the overall shape of each individual independent of left–right asymmetry, capturing variation in traits such as relative width, length, or overall carapace form. In contrast, the asymmetric component isolates the differences between the right and left sides of the carapace, thereby representing deviations from bilateral symmetry. Principal Component Analyses (PCA) were performed on both components to explore patterns of morphological variation among sites according to symmetric and asymmetric variation. Subsequently, discriminant function analyses were conducted between all site pairs to evaluate differences in symmetric and asymmetric components. Finally, using R and RStudio with the *lme4* package (Bates et al,. 2015), a generalized linear mixed model was fitted using the gamma family and assigning site as a random effect, after previously calculating VIF values and retaining only variables with values below 10.

2.1.2 Results.

Specimen Collection and Site Characterization

A total of 50 *H. bouvieri* individuals were collected across all municipalities. On average, each site contributed 10 individuals, with the exception of Guadalupe, and Rionegro, where 7 and 13 individuals were recorded respectively. All specimens were deposited in the Natural History Museum collection of the Industrial University of Santander (UIS-MHB). The five municipalities differed both in their physicochemical variables and their HII, indicating the presence of an environmental gradient among municipalities. The HII values were higher in Rionegro (0.63) and Bucaramanga (0.63), followed by Piedecuesta (0.61), Santa Bárbara (0.53), and Guadalupe (0.41), significant differences between groups were found by a parametric ANOVA ($p < 0.05$ / F = 515.3).

The physicochemical variables exhibited a partially different pattern. Bucaramanga presented the highest values of EC (529 µS/cm) and total dissolved solids (262.5 ppm), followed by Rionegro and Piedecuesta. In contrast, Santa Bárbara recorded the lowest conductivity (99.6 µS/cm) and total dissolved solids (48.8 ppm) values, whereas Guadalupe exhibited intermediate values for these variables. The two variables, EC and TDS were submitted to a parametric ANOVA and there were significant differences between the groups of each variable ($p < 0.05$) (EC F value = 84.68 / TDS F value = 75.38). The pH presented a similar pattern, with Rionegro having the highest (8.22) followed by Bucaramanga (8.12) and Piedecuesta (7.53). However, Santa Bárbara and Guadalupe exhibited the lowest values (7.45 and 7.29 respectively) (Table 1). The differences between these groups were also tested with a parametric ANOVA obtaining a significant difference ($p < 0.05$ / F = 13.85). The water temperature didn't show any variation among the groups.

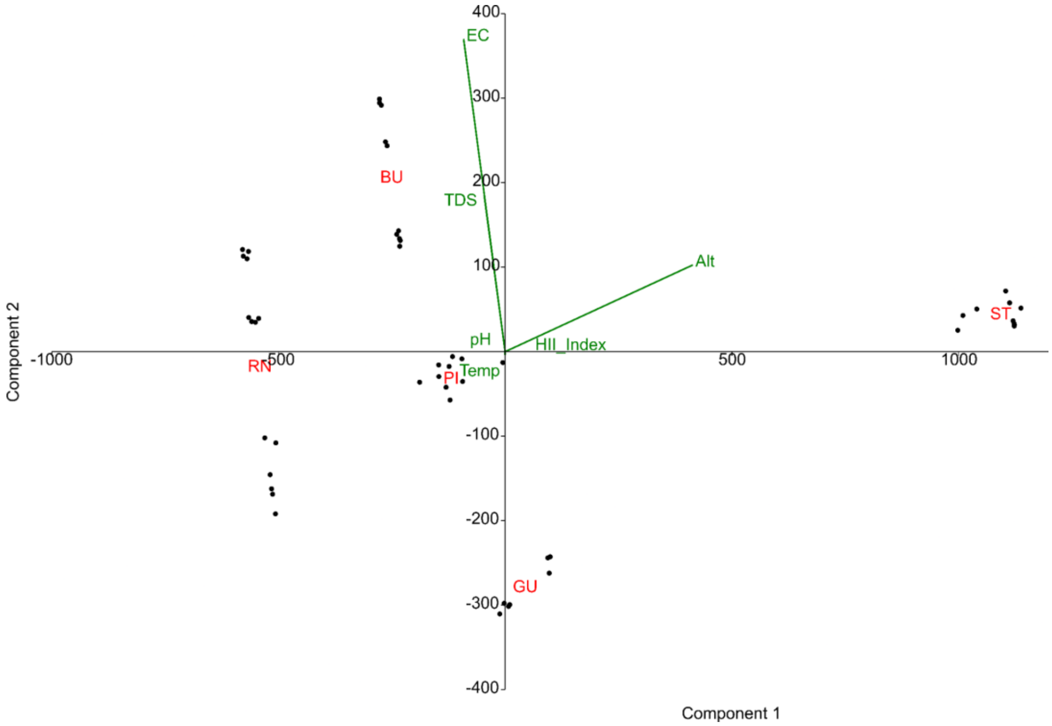
Table 1 – Physicochemical measure for each municipality

	pH	Temp (°C)	EC (µS/cm)	TDS (ppm)	HII	Alt (masl)
Site						
Bucaramanga	8.12±0.53	18.6±0.84	529±71.72	262.5±33.5	0.63±0.01	1050±1.9
Piedecuesta	7.53±0.16	21.2±0.42	297.2±13.87	147.2±6.3	0.61±0.01	1136.7±44.3
Rionegro	8.22±0.32	19.3±1.08	395.4±108.6	200±59.9	0.63±0.01	714.8±8.2
Santa Bárbara	7.45±0.38	18.8±1.03	99.6±16.4	48.8±6	0.52±0.05	2313.8±51
Guadalupe	7.29±0.29	22.1±1.21	44.2±14.7	22±7.3	0.4±0.01	1219±57

NOTE: The median and standard deviation for each variable measured in the sites. EC (Electrical conductivity); TDS (Total dissolved solids); HII (Habitat Integrity Index); Alt (Altitude)

To summarize environmental variation among localities, a Principal Component Analysis (PCA) was performed using physicochemical variables, HII, and altitude. The first two principal components explained 99.93% of the total variance, with PC1 accounting for 94.0% and PC2 for 5.93%. PC1 was primarily associated with altitude and, to a lesser extent, HII, whereas PC2 was mainly driven by EC and TDS. The PCA revealed a clear separation among municipalities. Santa Bárbara was located on the positive side of PC1 and was associated with higher altitude, while Rionegro occupied the opposite side of the axis. Along PC2, Bucaramanga was associated with higher EC and TDS values, whereas Guadalupe was located on the negative side of the axis and characterized by lower values of these variables. Piedecuesta occupied an intermediate position near the center of the ordination space (Figure 3). The study sites exhibited an environmental gradient, with Bucaramanga and Rionegro characterized by more disturbed environmental conditions, Santa Bárbara and Guadalupe representing comparatively less disturbed localities, and Piedecuesta occupying an intermediate position along the gradient.

Figure 3 – PCA used to summarize site characterization with physicochemical variables, HII and altitude



NOTE: GU (Guadalupe); BU (Bucaramanga); ST (Santa Bárbara); PI (Piedecuesta); RN (Rionegro)

Fluctuating Asymmetry and Shape Variation

The Procrustes ANOVA revealed significant effects of both directional asymmetry and fluctuating asymmetry in *H. bouvieri* (*ind* × *side*, $p < 0.05$; *side*, $p < 0.05$). Additionally, the mean square (MS) associated with the individual x side interaction exceeded the measurement error mean square, indicating that asymmetry variation was primarily biological rather than attributable to digitizing error. The significant side effect indicates consistent differences between left and right sides of the carapace (directional asymmetry), whereas the significant individual × side interaction reflects random deviations from bilateral symmetry among individuals (fluctuating asymmetry). Furthermore, the site effect was significant (*extra1*, $p < 0.05$), indicating that overall carapace shape differed among localities (Table 2).

Table 2 - Measurement error Procrustes Anova for both centroid size and carapace shape in H. bouvieri

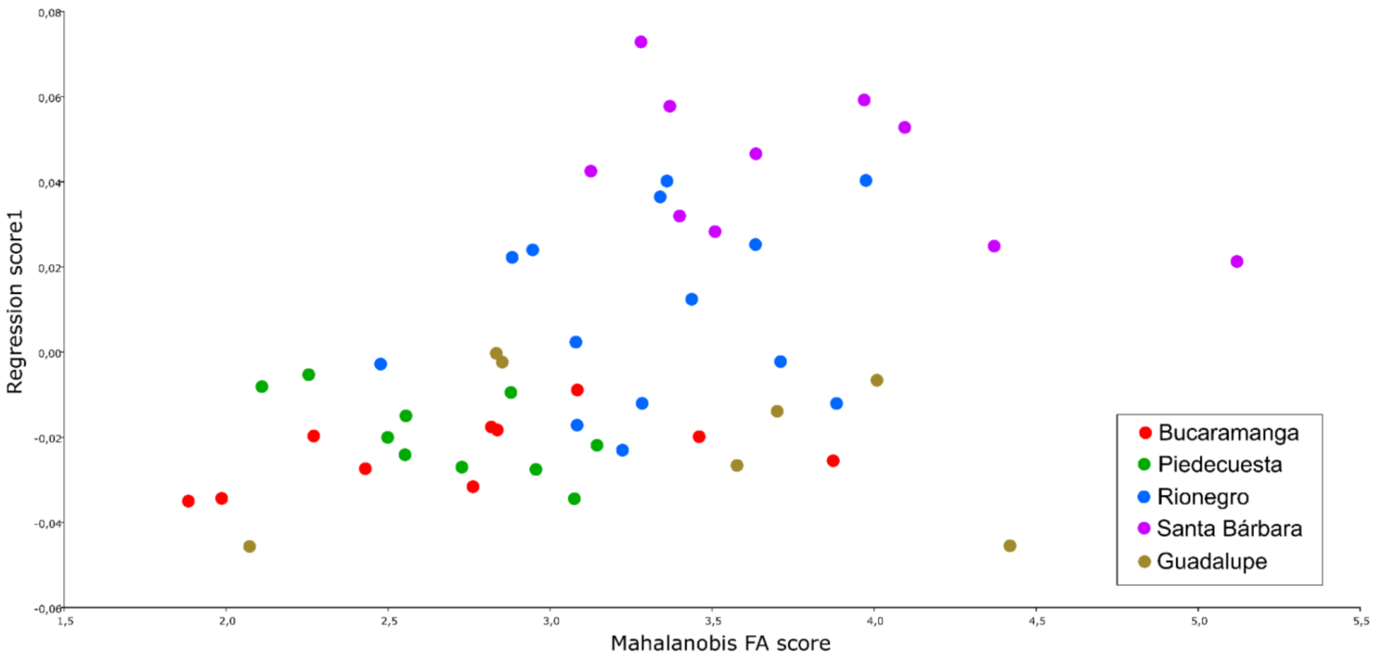
Centroid Size	SS	MS	d.f	F	p (param.)
Effect					
Extra 1 (Site)	156.78983	39.19746	4	15.54	<.0001
Individual	116.03467	2.52249	46	8.91	<.0001
Error 1	**14.43091**	**0.28296**	51		
Shape, Procrustes ANOVA:					
Effect	SS	MS	d.f	F	p (param.)
Extra 1 (Site)	0.086584	0.001273	68.00	7.62	<.0001
Individual	0.130683	0.000167	782.00	4.78	<.0001
Side	0.014579	0.000858	17.00	24.55	<.0001
Ind * Side	0.029692	0.000035	850.00	1.83	<.0001
Error 1	**0.033149**	**0.000019**	**1734.00**		

NOTE: SS (Sum of Squares); MS (Median Square); d.f (Degrees of Freedom); Side (Directional Asymmetry); Ind*Side (Fluctuating Asymmetry); Extra 1 (Site effect)

Individual Mahalanobis asymmetry values associated with each specimen showed that Bucaramanga (3.03) and Santa Bárbara (3.13) presented the highest average levels of fluctuating asymmetry, followed by Rionegro (2.83), Piedecuesta (2.48), and finally Guadalupe (2.65). A one-way parametric ANOVA detected significant differences in asymmetry levels among municipalities ($p < 0.05$ / F = 4.099).

The regression between shape and individual mahalanobis asymmetry values revealed the formation of two major point clusters: the first comprising individuals from Bucaramanga, Piedecuesta, Guadalupe, and a small number of specimens from Rionegro ; and the second including all individuals from Santa Bárbara and the remaining Rionegro specimens (Figure 4). A significant relationship was detected between individual Mahalanobis asymmetry values and shape variation ($p < 0.05$), indicating that changes in shape were associated with fluctuations in asymmetry. However, variance predicted was only 7.71% between the asymmetry values and the shape. The distribution of individuals along the regression scores revealed a clear pattern among sites. Santa Bárbara exhibited the highest regression score values and was associated with higher levels of fluctuating asymmetry. In contrast, Bucaramanga and Piedecuesta showed the lowest regression scores and lower asymmetry values. Rionegro occupied an intermediate position between these groups, whereas individuals from Guadalupe displayed a more dispersed distribution across the regression space.

Figure 4 – Regression between shape and fluctuating asymmetry individual values.



Two PCA analyses were performed, one considering the symmetric component and another the asymmetric component. For the symmetric component, PC1 explained 45% of the variance, PC2 explained 15%, and PC3 explained 12%. For the asymmetric component, PC1 explained 35% of the variance, PC2 explained 19%, and PC3 explained 10%. The scatterplot generated from the PCA of the symmetric component revealed two distinct point clouds and similar with the anova: one grouping Bucaramanga, Piedecuesta, Guadalupe, and a few Rionegro individuals, and another grouping Santa Bárbara specimens together with the remaining Rionegro individuals, meaning that there are two groups with different shapes (Figure 5). In contrast, the PCA of the asymmetric component showed a wide overlap between each municipality, showing that municipalities share the asymmetry patterns (Figure 6).

Figure 5 – PCA using the symmetric component

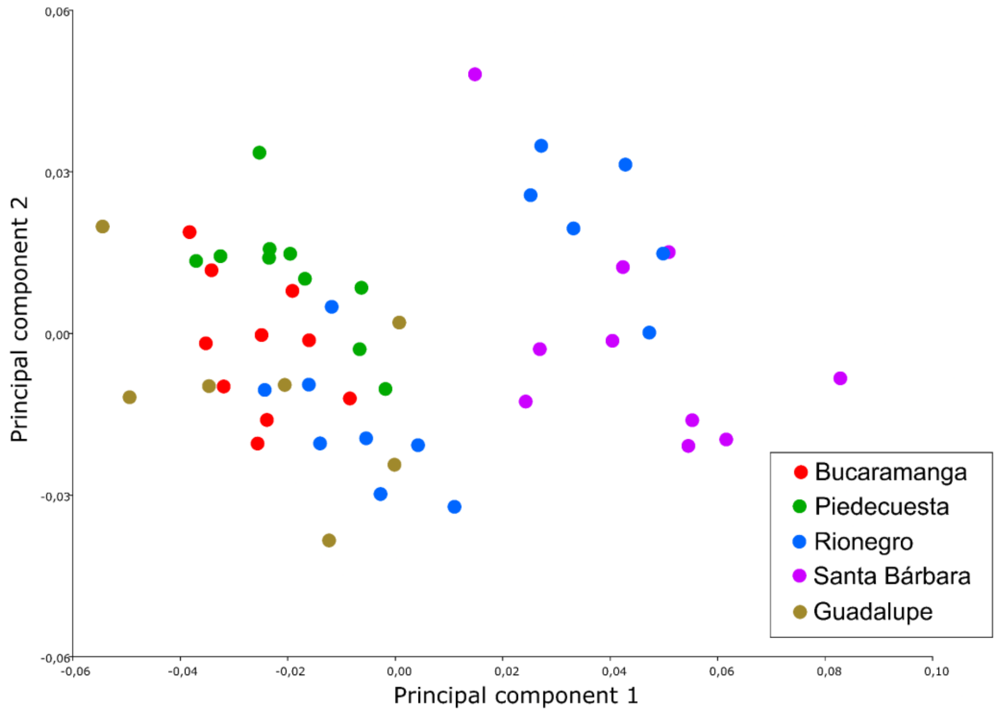
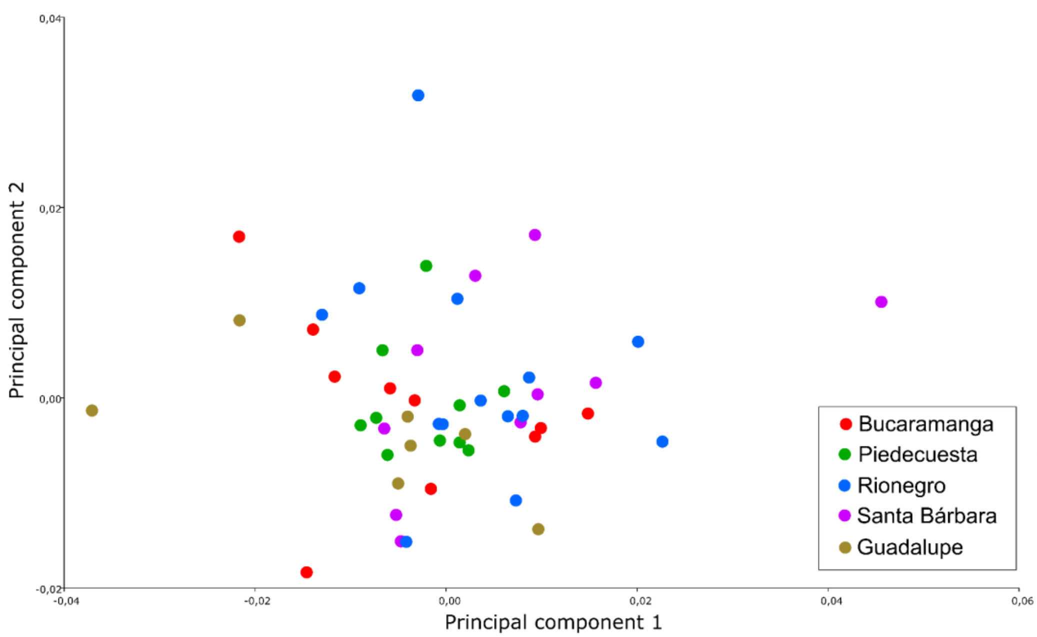
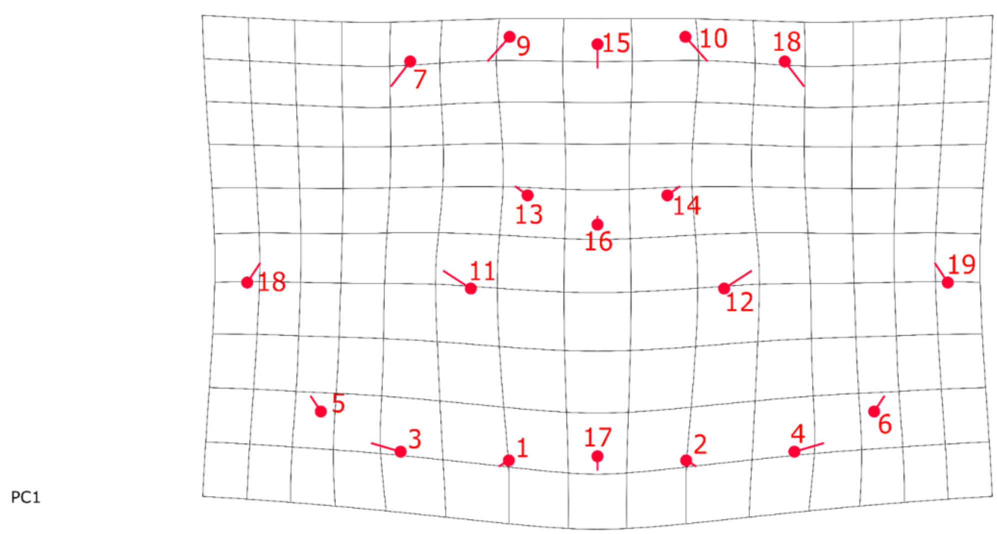


Figure 6 – PCA using the asymmetric component



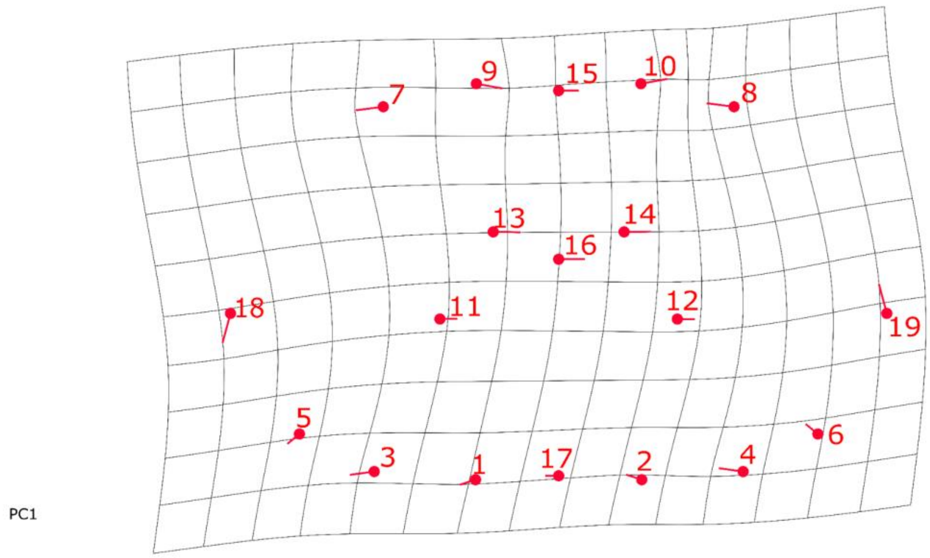
Deformation grids based on PC1 were generated for both the symmetric and asymmetric PCA components to visualize landmark variation associated with the main axis of shape variation. The deformation grid of the symmetric component revealed outward and upward displacements of landmarks 7, 8, 9, and 10. Similarly, landmarks 1, 2, 3, and 4 showed lateral expansion, while landmarks 18 and 19, corresponding to the lateral carapace margins, exhibited a marked outward displacement. In contrast, the central region of the carapace, represented by landmarks 13, 14, 15, 16, and 17, showed minimal deformation. These patterns suggest that symmetric variation is primarily associated with changes in overall carapace width, resulting from either lateral expansion or contraction of the carapace margins (Figure 7).

Figure 7 – Deformation Grid using the symmetric component



The deformation grid of the asymmetric component revealed a different pattern. Landmarks 7, 8, 9, 10, and 15 exhibited slight lateral displacements, whereas landmarks 11, 12, 13, 14, and 16, located in the branchial chamber region, remained comparatively stable. The most pronounced asymmetrical shifts were observed in landmarks 18 and 19, corresponding to the lateral margins of the carapace. Additionally, landmarks 1, 2, 3, 4, 5, 6, and 17 displayed minor lateral displacements. Together, these deformations suggest a subtle lateral twisting of the carapace, with asymmetrical variation concentrated primarily along the lateral margins rather than in the central region (Figure 8).

Figure 8 – Deformation Grid using the asymmetric component



Discriminant function analysis revealed significant differences among most sampling sites. For the asymmetric component, Guadalupe and Bucaramanga did not differ significantly (p = 0.2216), nor did Bucaramanga and Piedecuesta (p = 0.6945), whereas all remaining pairwise comparisons were significant ($p < 0.05$). In contrast, the symmetric component showed significant differences among all sites ($p < 0.05$). Generalized linear models indicated that individual asymmetry scores were not significantly associated with either environmental variables or the Habitat Integrity Index (HII), several models were fitted using different combinations of predictor variables ($p > 0.05$), suggesting that the environmental factors evaluated in this study did not play a major role in shaping fluctuating asymmetry in *H. bouvieri*.

2.1.2.1 Discussion.

A geometric morphometric approach was employed to assess the fluctuating asymmetry among the five collect sites of *H. bouvieri*. Shape variation was detected among sites; however, it was not associated with any of the environmental factors measured on the field. Although the exact causes underlying the pattern observed in this study remain uncertain, studies in other animal groups (Hampton et al., 2014; Lens & Van Dongen, 2000) have shown that morphological variation resulting from anthropogenic environmental modification may affect species differently (H. A. Benítez, Lemic, Bažok, Bravi, et al., 2014; H. A. Benítez, Lemic, Bažok, Gallardo-Araya, et al., 2014; Clarke, 1993; Lens & Van Dongen, 2000). Previous studies on crustaceans have reported contrasting responses of fluctuating asymmetry to environmental stressors. For instance, Fray (1992) observed no significant differences in fluctuating asymmetry among crayfish of the genus *Orconectes* exposed to heavy metal contamination. Similarly, Fuller et al. (2022) found no evidence of increased fluctuating asymmetry in populations of the freshwater crab *Eriocheir japonica* inhabiting areas with differing levels of radiocesium contamination. In contrast, Frota et al. (2019) reported higher levels of fluctuating asymmetry in populations of the coastal crustacean species, *Atlantorchestoidea brasiliensis* and *Emerita brasiliensis* from disturbed environments, whereas individuals from better preserved habitats showed lower levels. These contrasting findings highlight the variable nature of fluctuating asymmetry responses in crustaceans and suggest that developmental instability does not necessarily exhibit a direct or consistent relationship with environmental stressors.

Differences in the carapace of *H. bouvieri* were detected in all landmarks of the symmetric component, suggesting that the variation patterns are primarily associated with the carapace width, resulting in lateral expansion or contraction of the margins. Likewise, the asymmetric component exhibited variation across all landmarks, showing that asymmetrical changes were concentrated mainly along the lateral margins of the carapace, while the central region remained comparatively stable, this suggests the presence of a subtle lateral twisting pattern in the carapace. Individuals from Santa Bárbara exhibited the highest Mahalanobis asymmetry values among all sampled municipalities. Although the environmental variables measured at this site did not differ markedly from other localities, the symmetric component PCA showed that Santa Bárbara individuals occupied a distinct region of the morphospace. Likewise, the Santa Bárbara population exhibited a distinct shape pattern, separating it from the remaining populations. In contrast, specimens from Bucaramanga, Piedecuesta, Guadalupe, and Rionegro clustered together, forming a well-defined group of shape variation. This pattern suggests the existence of two major morphological configurations: one associated with Santa Bárbara and some individuals from Rionegro, and another associated with the remaining sites. However, this separation was only evident when considering the symmetric component, reflecting differences in overall shape. In contrast, analysis of the asymmetric component revealed no clear separation among municipalities, with specimens of all sites clustering together. This result indicates that asymmetry patterns are largely shared among populations and are not specific to any particular site.

This study represents the first morphometric assessment of the carapace shape variation in a Pseudothelphusidae species, demonstrating that carapace shape varies among localities. Using the symmetric component, a consensus shape was generated from all study sites and subsequently compared with the mean shape of each locality. Among all populations, Santa Bárara exhibited the most pronounced morphological deviation from the overall consensus shape, showing noticeable landmark displacements along the lateral margins (landmarks 18, 19, 5, and 6), as well as in the anterior and posterior regions of the carapace (landmarks 1, 2, 7, 8, 9, 10, 15, and 17) (Appendix P).

These shape changes indicate a generalized modification of carapace morphology relative to the overall mean configuration. In contrast, comparisons between the consensus shape and the mean shapes of the remaining localities revealed only minor landmark displacements, with no substantial deviations from the overall configuration (Appendix L, M, N, O). This pattern suggests that the Santa Bárbara population possesses a distinctly different carapace morphology relative to the other study sites. The shape differentiation observed in Santa Bárbara may be associated with the particular environmental conditions of this locality, especially its higher elevation relative to the other study sites. High elevation environments are characterized by lower partial oxygen pressure, which can represent a significant elevation physiological challenge for aquatic organisms. In crustaceans, limitations in oxygen availability have been linked to changes in morphology and development of structures involved in gas exchange (Hawkins et al., 1982). In this context, the distinct shape observed in individuals from Santa Bárbara may be reflecting a response to these environmental conditions (Ivleva, 1980; Vargas et al., 2010). Although the present study did not directly evaluate respiratory variables or physiological mechanisms, these may be a possible explanation to the shape differentiation between Santa Bárbara and the other localities evaluated. Likewise, the observed differentiation may reflect geographic separation among populations, resulting in morphological differentiation through restricted gene flow or local adaptation. Although several Pseudothelphusidae species exhibit narrow ecological niches and highly restricted distributions, *H. bouvieri* displays the opposite pattern, occurring across a broad altitudinal and latitudinal range and inhabiting environments with varying levels of disturbance. This ecological resilience and its ability to persist across diverse habitats may help explain the lack of variation observed in the asymmetric component.

3. Conclusions

The present study constitutes the first assessment of fluctuating asymmetry in *Hypolobocera bouvieri* using geometric morphometric techniques and provides the first evaluations of developmental instability in a pseudothelphusid crab under contrasting environmental conditions in Colombia. The environmental characterization of the study sites revealed the existence of environmental gradients among localities. Differences were detected in the habitat integrity index and physicochemical conditions, with sites exhibiting contrasting combinations of conductivity, total dissolved solids, altitude, and Habitat Integrity Index values. However, these environmental variables did not vary in a strictly concordant manner, indicating that no single variable adequately described the degree of environmental disturbance across all localities.Geometric morphometric analyses successfully quantified fluctuating asymmetry in *H. bouvieri*. The Procrustes ANOVA detected significant fluctuating asymmetry and directional asymmetry, demonstrating the presence of measurable developmental instability within the studied sites. Shape variation was concentrated primarily along the lateral margins of the carapace, which represented the most morphologically variable region in both the symmetric and asymmetric components of shape variation. Morphological differences among sites were evident, particularly in the symmetric component of shape variation. Individuals from Santa Bárbara and Rionegro formed a distinct morphological group, whereas specimens from Bucaramanga, Piedecuesta, Guadalupe, and part of Rionegro exhibited a more similar morphology.

These results suggest the existence of geographic variation in carapace shape among the different sites where *H. bouvieri* was collected, potentially associated with local environmental conditions. Despite the presence of significant fluctuating asymmetry and morphological differentiation among sites, no significant relationship was detected between individual asymmetry scores and the environmental variables measured in this study. Similarly, habitat integrity and physicochemical characteristics did not explain the observed patterns of fluctuating asymmetry. These findings suggest that developmental instability in *H. bouvieri* may be influenced by factors other than those evaluated here, or that this species possesses a degree of ecological resilience that reduces the impact of environmental variation on developmental stability. Overall, this study provides a novel baseline for future research on developmental stability, morphological variation, and environmental responses in Neotropical freshwater crabs. The results contribute to the ecological knowledge of *H. bouvieri* and highlight the value of geometric morphometrics as a tool for investigating morphological variation and fluctuating asymmetry in freshwater crustaceans.

4. Recommendations

Future studies should increase sampling size among all localities to improve statistical representation and robustness, providing a more accurate morphological variation of the population. A large number of specimens would allow a precise evaluation on fluctuating asymmetry patterns and may reduce potential biases. Also, it is recommended that future research should expand the geographic coverage of sampling to include additional Andean micro water-sheds and populations across broader altitudinal and latitudinal gradients. This may allow a more comprehensive assessment of the spatial variation and facilitate comparisons among the populations, even some populations that may be exposed to different environmental pressures. Additional environmental variables should be measured, including dissolved oxygen, substrate composition and even concentrations of potential pollutants such as heavy metal or agriculture residuals. Even, future analyses may incorporate molecular approaches, like population genetics or phylogeographic analyses, to evaluate whether the morphological differences observed among the sites are associated to genetic structure or local adaptation processes. Finally, constant monitoring programs would be accurate to establish to evaluate temporal variation in fluctuating asymmetry and determine if the morphological changes remain stable or change under certain anthropogenic influence or environmental transformation.

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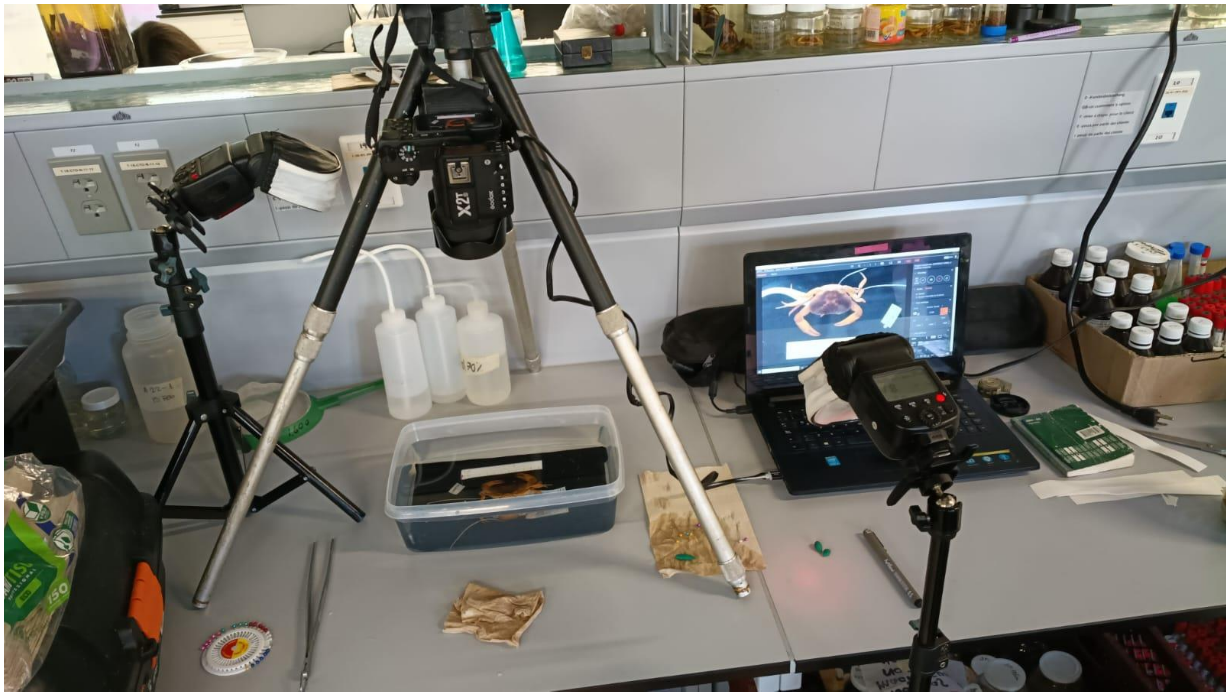
Appendix



Appendix A – Setup for photography on each specimen

Appendix B – Bucaramanga Collection Site

Appendix C – Guadalupe Collection Site 1

Appendix D – Guadalupe Collection Site 2

Appendix E – Piedecuesta Collection Site

Appendix F – H. bouvieri collected on Piedecuesta

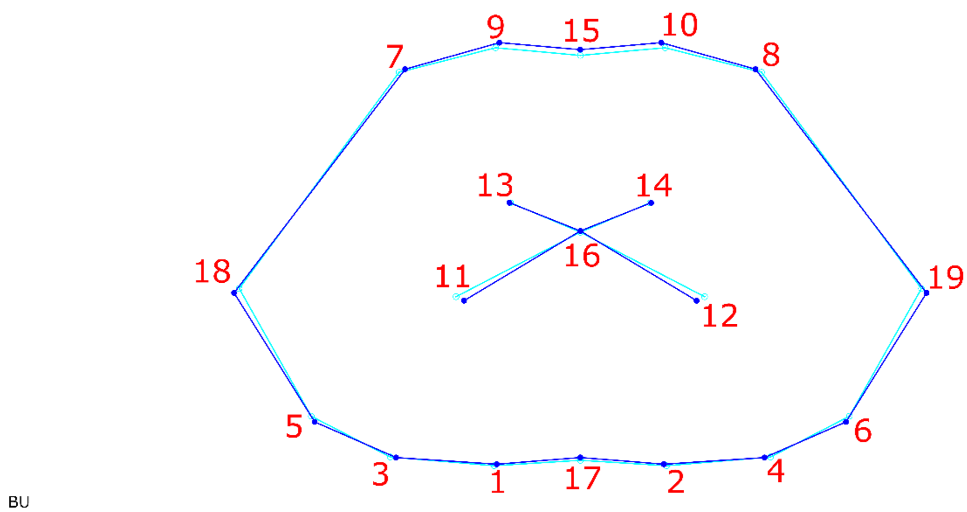
Appendix G – Rionegro collection site 1

Appendix H – Rionegro collection site 2

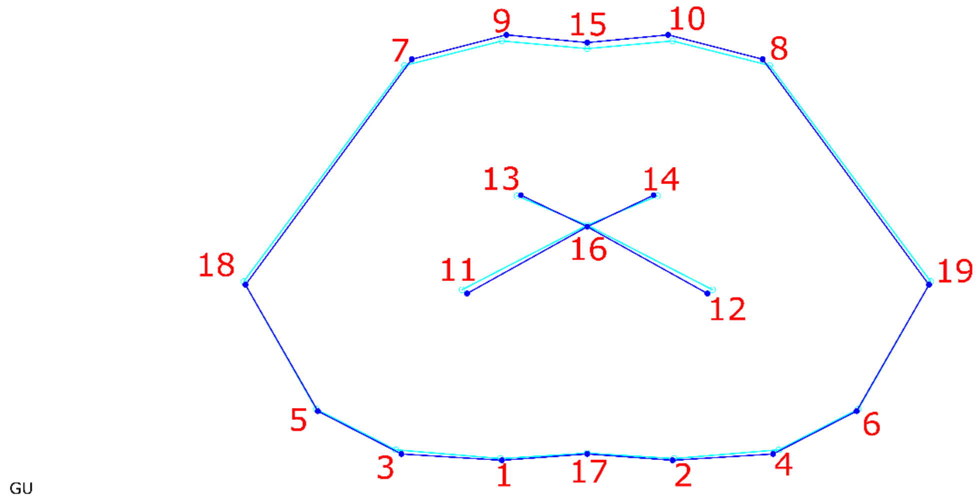
Appendix I – H. bouvieri collected in Rionegro

Appendix J – Santa Bárbara Collection site 1

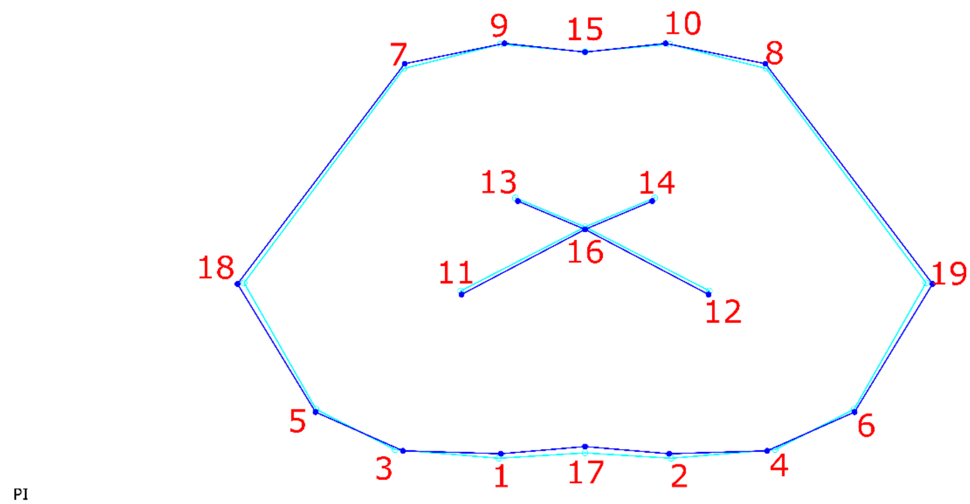
Appendix K – Santa Bárbara Collection site 2



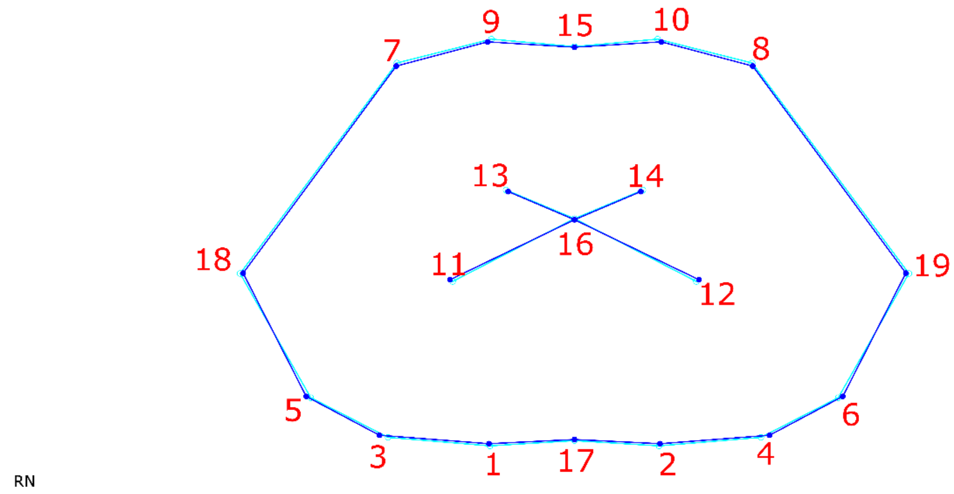
Appendix L – Total localities mean shape (light blue) vs. Bucaramanga shape (dark blue)



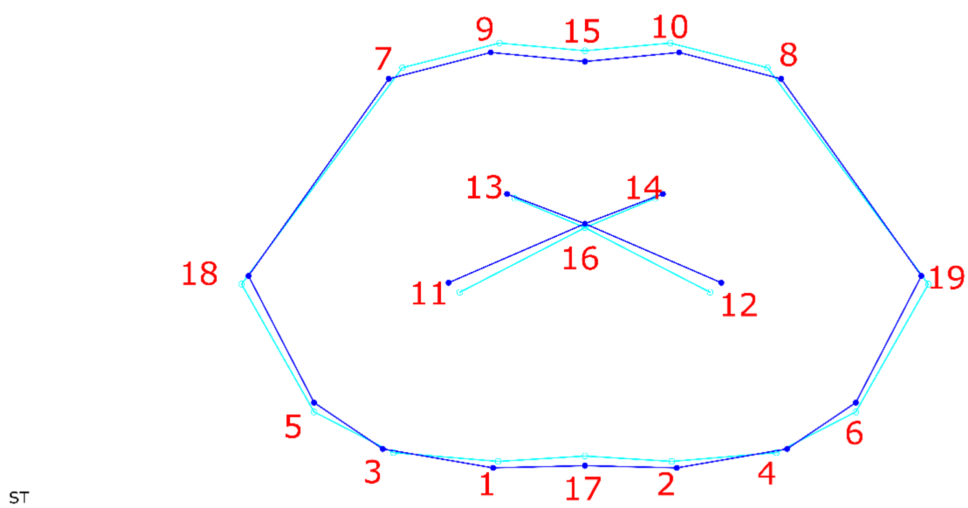
Appendix M – Total localities mean shape (light blue) vs. Guadalupe shape (dark blue)



Appendix N – Total localities mean shape (light blue) vs. Piedecuesta shape (dark blue)



Appendix O – Total localities mean shape (light blue) vs. Rionegro shape (dark blue)



Appendix P – Total localities mean shape (light blue) vs. Santa Bárbara shape (dark blue)

ID	Site	FA_Score	pH	TDS	EC	Temp	HII_Index	Alt
hypH001BU02	BU	4.156149	7.62	300	610	19	0.62	1051.4
hypH002BU01	BU	2.650824	8.12	234	468	19	0.65	1047.7
hypM001BU01	BU	3.21234	8.79	230	460	19	0.65	1053.4
hypM002BU01	BU	3.26916	8.24	232	462	19	0.65	1052.1
hypM003BU01	BU	3.432146	8.56	237	470	19	0.65	1052.5
hypM004BU01	BU	2.974666	8.81	228	454	19	0.65	1050.6
hypM005BU02	BU	2.071591	7.22	302	613	19	0.62	1048.2
hypM006BU02	BU	2.735638	7.54	308	615	19	0.62	1049.2
hypM007BU03	BU	3.079961	8.09	275	567	17	0.63	1052.2
hypM008BU03	BU	2.728387	8.24	279	571	17	0.63	1049.8
hypH001GU01	GU	2.600211	7.14	17	34	23	0.4	1184.2
hypM001GU02	GU	3.319144	7.05	23	48	23	0.42	1277.7
hypM002GU01	GU	1.893396	7.83	19	38	23	0.4	1172.6
hypM003GU01	GU	2.834246	7.57	16	33	23	0.4	1181
hypM004GU02	GU	2.794128	7.16	32	64	23	0.42	1279
hypM005GU02	GU	2.388358	7.21	32	64	23	0.42	1284.3
hypM006GU01	GU	2.552393	7.07	15	29	23	0.4	1160.1
hypH001PI01	PI	2.074191	7.84	154	308	21	0.61	1114.6
hypH002PI02	PI	2.806175	7.55	148	296	22	0.59	1118.8
hypH003PI01	PI	2.750585	7.24	152	310	21	0.61	1060.3
hypH004PI03	PI	2.360831	7.49	146	289	21	0.63	1126.2
hypM001PI01	PI	2.072258	7.68	142	308	21	0.61	1138.5
hypM002PI01	PI	2.288341	7.57	156	312	21	0.61	1141.3
hypM003PI01	PI	3.178154	7.65	153	306	21	0.61	1156.5
hypM004PI02	PI	2.563496	7.45	140	287	22	0.59	1146.8
hypM005PI03	PI	2.4996	7.52	138	270	21	0.63	1126.6
hypM006PI03	PI	2.243809	7.39	143	286	21	0.63	1238.2
hypH001ST02	ST	3.399644	7.18	54	118	18	0.52	2268.1
hypH002ST02	ST	3.264528	7.12	50	104	18	0.52	2221.4
hypH003ST02	ST	3.398103	7.15	48	96	18	0.52	2362.9
hypM001ST01	ST	2.966	7.93	41	84	20	0.53	2343.7
hypM002ST01	ST	3.238261	7.98	42	80	20	0.52	2343.5
hypM003ST01	ST	2.708551	7.81	47	85	20	0.53	2342.4
hypM004ST01	ST	3.127733	7.89	43	81	20	0.53	2344.1
hypM005ST02	ST	2.5957	7.12	54	118	18	0.53	2236.6
hypM006ST02	ST	3.59517	7.18	49	109	18	0.53	2340.2
hypM007ST02	ST	3.050992	7.17	60	121	18	0.53	2335.2
hypH001RN02	RN	2.39674	8.02	126	326	19	0.63	729.4
hypM001RN01	RN	2.899894	8.45	236	438	19	0.63	720.1
hypM002RN01	RN	2.43156	8.41	240	439	18	0.63	728.2
hypM003RN01	RN	2.831189	8.43	242	445	18	0.63	707.1
hypM004RN01	RN	2.681002	8.67	238	440	18	0.63	712.3
hypM005RN02	RN	2.503977	7.82	158	323	19	0.66	707.1
hypM006RN01	RN	2.240117	8.85	260	521	19	0.63	725.3
hypM007RN01	RN	3.206546	8.22	257	514	20	0.63	720.2
hypM008RN02	RN	2.981717	7.87	120	238	21	0.66	708.5
hypM009RN02	RN	3.157033	7.83	135	265	21	0.66	707.2
hypM010RN02	RN	2.520257	8.03	130	260	21	0.63	707.9
hypM011RN01	RN	3.031256	8.34	262	526	19	0.63	713.2
hypM012RN01	RN	3.577583	8.27	258	519	19	0.63	712.9
hypM013RN02	RN	3.282833	7.92	139	282	20	0.66	708.2

Appendix Q – Main table with FA values and Physicochemical variables for each individual