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# Quantifying wing pattern sexual dimorphism in the butterfly *Anartia amathea roeselia* Eschscholtz (Lepidoptera: Nymphalidae)

**Ronaldo Bastos Francini, Thadeu Sobral Souza, Renato Rogner Ramos, Maria Sylvia Cerávolo**

## Abstract
The present study searched for morphological markers in the wings of *Anartia amathea roeselia* butterfly. This species can be used as a model species due to its natural abundance in SE Brazil and Neotropics. It is an excellent tool for analysis of phenotypical traits and in studies of population genetics. In this research, wing pattern of both sexes were described and quantified in a sample of 812 butterflies considering sexual dimorphism related to size and colour. The results revealed that approximately 55% of the marker spots on the dorsal surface of wings presented little (< 2.5%) or no variation. Therefore, only ten of 22 spots were used as markers in the analysis, with some spots being sexually dimorphic. The female size was bigger than males as well as other Nymphalidae species. The results showed that the colour wing pattern of *A. a. roeselia* had enough differences to separate individuals of both sexes and morphological markers that can be used to study the role of natural selection upon populations of this species.

**Keywords:** Colour pattern, sexual selection, natural selection

O presente estudo pesquisou marcadores morfológicos nas asas da borboleta *Anartia amathea roeselia*. Esta espécie pode ser usada como modelo devido à sua abundância natural nos neotrópicos e na região sudeste do Brasil. É uma excelente ferramenta para a análise de caracteres fenotípicos e no estudo de genética de populações. Por isso, os padrões alares dos dois sexos foram descritos e quantificados em uma amostra de 812 borboletas, considerando o dimorfismo sexual relacionado ao tamanho e a cor. Os resultados revelaram que aproximadamente 55% das machas marcadoras na superfície dorsal das asas apresentaram pouca (< 2,5%) ou nenhuma variação. Por isso, só 10 das 22 foram utilizadas nas análises, sendo algumas delas sexualmente dimórficas. O tamanho das fêmeas foi maior que o dos machos como em outras espécies de Nymphalidae. Os resultados mostraram que o padrão de cor das asas de *A. a. roeselia* tem diferenças suficientes para separar indivíduos dos dois sexos e que os marcadores morfológicos podem ser usados para estudar o papel da seleção natural sobre as populações dessa espécie.

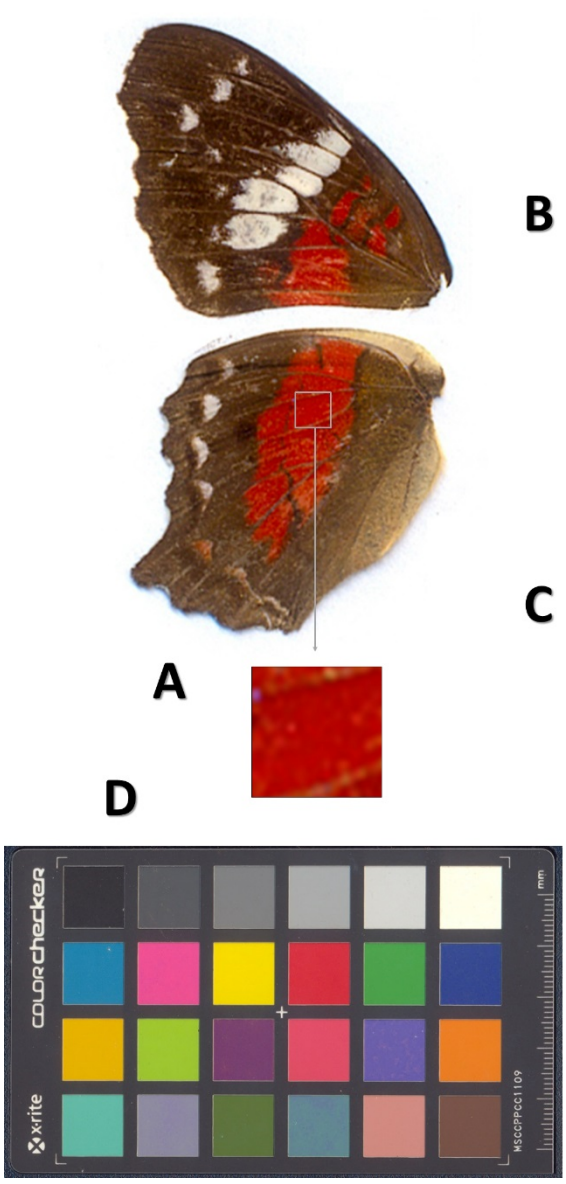
**Palavras-chave:** padrão de cor, seleção sexual, seleção natural

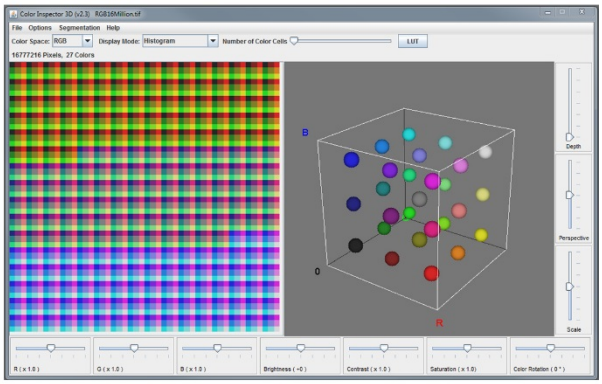
## 1. Introduction
The wings of butterflies contain enormous quantity of information and tell us the results of natural selection in a "graphical" manner [1, 2]. Environmental sensitivity during developmental stages [3, 4, 5, 6], thermoregulation [7, 8], and sexual selection [9] are factors involved in selection and development of wing patterns.
Species with continental distributions or along the climatic gradients can gain distinct features among populations [10, 11]. These features are size, shape, colour pattern and until behaviour [12]. Geographical distribution can shape hybrid zones, abrupt transitions, clines, or mosaics, all influenced by environmental standards, genetic factors, natural selection and by intrinsic characteristics of the species, forming geographical races [13].
However, the differences among populations not always follow these traditional patterns of distributions, sometimes they can occur in a small scale inside a population, e.g. differences among individuals or genders [14]. Therefore, independently of scale, population studies need that a large number of individuals be sampled.

Sexual dimorphism in butterfly wing pattern was studied at first time by Scudder [15] who used the word antigeny to explain morphological differences between genders as a by-product of sexual selection. Oliver & Monteiro [16] stated that sexual dimorphism is probably influenced by underlying genetic architecture responsible for sex-limited expression.
Due to availability of material, common species are indicated as models for studies involving phenotypical analysis of populations.

A common butterfly species in SE Brazil is *Anartia amathea roeselia* (Eschscholtz, 1821) (Nymphalidae: Nymphalinae: Nymphalini) which, despite of its commonness in neotropics, the only previous field study was made by Fosdick [17] at Ecuador.

DeVries [18] stated that in Costa Rica, butterflies of the genus *Anartia* Hübner, [18, 19] have a high chance of to be the first sited by a foreign visitor. Silberglied *et al.* [19] showed that in Neotropics populations of butterflies of the genera *Anartia* and *Junonia* Hübner, [18, 19] are common in environments where natural impacts or anthropogenic activities benefit them.
They also stated that their larval foodplants grow along the draining or irrigation channels in roads or cultivated fields where big populations can be found with frequent local extinctions. Observations made at Baixada Santista, São Paulo, Brazil in Quilombo River area [20] showed that this subspecies is abundant during autumn (April-May) where groups with more than 50 butterflies had been observed in patches with flowers of *Bidens alba* (Asteraceae). Due to the availability and abundance of this butterfly species, we have been preparing a series of research on different aspects of natural history, behaviour and ecology of *A. a. roeselia*. This one describes the wing pattern of both sexes; quantify their colours, and makes consideration on sexual dimorphism related to size and colour.



| R | G | B | FREQUENCY | % | COLOR HUE |
|---|---|---|---|---|---|
| 40 | 40 | 40 | 592704 | 3.533 | |
| 40 | 40 | 128 | 627984 | 3.743 | |
| 40 | 40 | 216 | 585648 | 3.491 | |
| 40 | 128 | 40 | 627984 | 3.743 | |
| 40 | 128 | 128 | 665364 | 3.966 | |
| 40 | 128 | 216 | 620508 | 3.699 | |
| 40 | 216 | 40 | 585648 | 3.491 | |
| 40 | 216 | 128 | 620508 | 3.699 | |
| 40 | 216 | 216 | 578676 | 3.449 | |
| 216 | 40 | 40 | 585648 | 3.491 | |
| 216 | 40 | 128 | 620508 | 3.699 | |
| 216 | 40 | 216 | 578676 | 3.449 | |
| 216 | 128 | 40 | 620508 | 3.699 | |
| 216 | 128 | 128 | 657443 | 3.919 | |
| 216 | 128 | 216 | 613121 | 3.654 | |
| 216 | 216 | 40 | 578676 | 3.449 | |
| 216 | 216 | 128 | 613121 | 3.654 | |
| 216 | 216 | 216 | 571787 | 3.408 | |
| 128 | 40 | 40 | 627984 | 3.743 | |
| 128 | 40 | 128 | 665364 | 3.966 | |
| 128 | 40 | 216 | 620508 | 3.699 | |
| 128 | 128 | 40 | 665364 | 3.966 | |
| 128 | 128 | 128 | 704969 | 4.202 | |
| 128 | 128 | 216 | 657443 | 3.919 | |
| 128 | 216 | 40 | 620508 | 3.699 | |
| 128 | 216 | 128 | 657443 | 3.919 | |
| 128 | 216 | 216 | 613121 | 3.654 | |

**Fig 1:** *Anartia amathea roeselia*. (A) Sampled square area in the hindwing red dorsal area of the butterfly. (B) Screen of the plugin colour 3D showing at left the 16,777,216 colour hues tiff file used to check the software and at right the 27 colour hues 3D graph.(C) LUT (look up table) showing the frequencies and percentages of main colour hues. (D) Color Checker Passport chart used to standardize the RGB colour.

## 2. Materials and methods

### 2.1 Study Area

Butterflies were collected in the valley of the Quilombo River (coordinates 23°51'35"S and 46°21'01"W to 23°49'18"S and 46°18'37"W) in the central coast of São Paulo, Brazil [20].

### 2.2 Samplings and processing

During autumn 2010, 11 samplings were made on March and April along the edges of the road, from 0900h to 1530h, within the period of activity these butterflies. Butterflies were netted and placed in tagged zip-loc envelopes that were maintained in an insulated box. In laboratory, the samples were dehydrated on air oven at 70 °C during a week and after placed in numbered zip-loc envelopes. After, left wings of each butterfly was isolated from body and placed in other zip-loc envelope with same tags. Males and females were separated based on sexual dimorphism, using wing characteristics or the morphology of the first pair of legs. Forewing length (FWL) (mm) was measured using a ruler with 0.5 mm precision and inspected both surfaces of wings to detect different traits between individuals of the same sex. Damaged wings were not considered. The presence or absence of coloured scales (e.g. white or yellow/orange scales) was observed at a level of 40 times magnification to confirm or not the presence of a spot. For quantification of colours, wings were scanned using an HP Scanjet G2710 scanner with 600 dpi resolution and high bit depth (12 bits). Resulting files were saved in TIFF format to maintain minimum loss of information. To standardize the colours obtained and minimize bias, we used a Color Checker Passport chart (Figure 1; D) with v. 1.0.2 software (X-Rite Inc.). A square area of ± 1 $mm^2$ (60 x 60 pixels) of the red area of the hindwing was chosen to be quantified (Figure 1 A). These samples were analysed extracting the pixel values of each RGB channel using ImageJ v. 1.44 p. software [21] with plug-in Color Inspector 3D, v. 2.3 [22] (Figure 1 B) which permitted to choose different combinations of colour classes. Colour distribution in the sample creates a Look Up Table (LUT) file with the frequencies and respective percentages of RGB values. We used the Colour Space = RGB and Display Mode = Histogram, adjusting the number of colour hues for the minimum. By pressing the button LUT, a list is created showing the absolute frequencies and respective percentages of each colour hue. When a standard file with 16,777,216 colour hues was used (Figure 1 C) the LUT minimum was 27 colour hues due to compression into classes. Comparisons between LUTs of the samples of two sexes were made using boxplot graphs for the three most frequent RGB classes: brown (128-40-40), red (216-40-40), and orange (216-128-40). All sampled material will also be used in other papers (manuscripts in preparation). Voucher material will be deposited in the collections of the ZUEC (Museu de Zoologia da Universidade Estadual de Campinas "Adão José Cardoso").

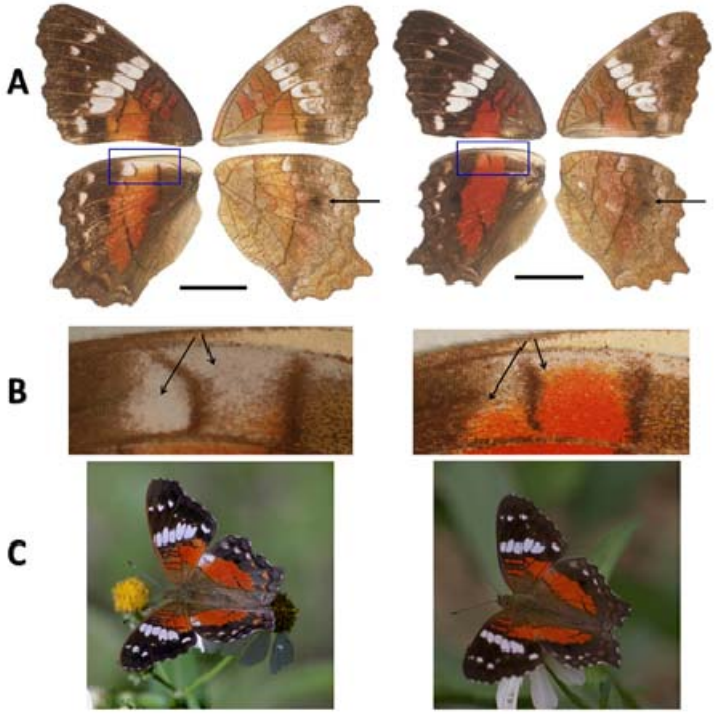



**Fig 2:** *Anartia amathea roeselia*. Basic colour pattern of wings. (A) Dorsal (left) and ventral (right) surfaces of female and male. Ventral surfaces are more pallid and present a cryptic pattern. Black arrow indicates the black hindwing spot, which is more visible on the ventral hindwing surface. Blue rectangle delimits sex dimorphic area. Scale bar = 10 mm. (B) Detail of sex dimorphic area where female has a white spot (spot 18; see Figure 3) and male red scales. (C) Actual landing behaviour of active female and male exposing the dimorphic area.

### 2.3 Data analysis

Basic data were tabulated using Excel with individuals placed in rows and wing traits in columns. R software v. 3.02 win [23] with package gMWT [24] were used to compare FWL between sexes using Kruskal-Wallis ANOVA with Wilcox test. Williams G test was used to compare sex ratio and wing spot frequencies.

## 3. Results

We analysed 812 butterflies, 282 being males and 530 females; sex ratio of 0.35:0.65 being significant different (Williams G test = 76.92; $p < 0.0001$). However, due to wing

damage of several butterflies the follow results have differences in totals. Basic colour pattern of wings of both sexes of the butterfly *A. a. roeselia* presents a conspicuous dorsal colour pattern but cryptic ventral colour pattern (Figure 2 A). When landed on flowers or heating in the sun, adults of both sexes open the wings parallel to substrate exposing the white spot on dorsal surface (Figure 2 B) that is sex dimorphic (Figure 2 C). Basic nymphalid wing groundplan is formed by three symmetry systems: basal, central and border [25, 26]. The wing pattern of *A. a. roeselia* described here will follow this scheme.

Basic colour pattern of wings. (A) Dorsal (left) and ventral (right) surfaces of female and male. Ventral surfaces are more pallid and present a cryptic pattern. Black arrow indicates the black hindwing spot, which is more visible on the ventral hindwing surface. Blue rectangle delimits sex dimorphic area. Scale bar = 10 mm. (B) Detail of sex dimorphic area where female has a white spot (spot 18; see Figure 3) and male red scales. (C) Actual landing behaviour of active female and male exposing the dimorphic area.

Basal symmetry system of dorsal area of both wings of adults of *A. a. roeselia* (Figure 3) is composed by two red/orange areas: the forewing red/orange area (Figure 3 FROA) and the hindwing red/orange area (Figure 3 HROA). In HW, close to abdomen there is a basal grey hair area (Figure 3 BHHA). On forewing and hindwing, respectively, there is a distal line of white spots (Figure 3 FWDS and HWDS). In the centre of forewing, there is a row of white patches (Figure 3 FWP). At basis, hindwing also presents three white, black and/or orange colour spots (Figure 3 HOSA) and between subcostal vein and wing border a sex dimorphic area (Figure 3 SDA).

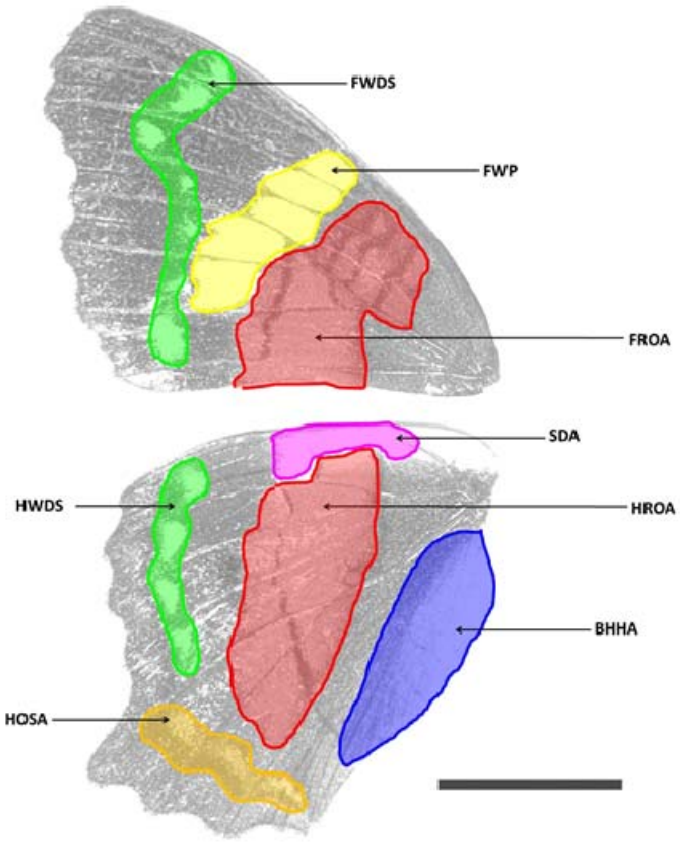



**Fig 3:** *Anartia amathea roeselia*. Elements of female colour pattern of wings. FWDS: forewing white distal spots; HWDS: hindwing white distal spots; FWP: forewing white patches; FROA: forewing red area; HROA: hindwing red area; HOSA: hindwing colour spots area; BHHA: basal hair hindwing area; SDA: sex dimorphic area. Scale bar = 10 mm.

Elements of female colour pattern of wings. FWDS: forewing white distal spots; HWDS: hindwing white distal spots; FWP: forewing white patches; FROA: forewing red area; HROA: hindwing red area; HOSA: hindwing colour spots area; BHHA: basal hair hindwing area; SDA: sex dimorphic area. Scale bar = 10 mm.

Area FWDS is composed by seven white spots (Figure 4, spots 1-7) and HWDS by four white spots (Figure 4, spots 8-11). Five or six white patches (Figure 4, patches 12-17) compose area FWD. At SDA females present a white spot (Figure 4, spot 18). At FROA there four red patches (Figure 4, patches D-G) and at HROA only one (Figure 4, patch H). Inside FROA there are two vertical black lines, the first in the middle and the second at edge (Figure 4, line FWVBL). They are continuous at HROA (Figure 4, line HWVBL). Area HOSA presents three colour spots (Figure 4, spots A-C). Long hairs cover the BHHA (Figure 4, area BHHA) which are exposed at near vertical position when the butterfly is landed with wings open (Figure 5 AB). Black hindwing spot (Figure 4, spots BHS) is more visible on the ventral surface, although being more apparent in females due to the weak intensity of the colour pattern when compared to males. colour intensity of FROA and HROA of males is higher than females (Figure 6 MF). Comparison between square areas of 100 $mm^2$ from the

hindwing red area of females and males of the butterfly *A. a. roeselia* showed that two classes brown and orange of the three most frequent colour hues: brown (128-40-40), red (216-40-40), and orange (216-128-40) were significant different (Figure 7). However, the classes of the three most frequent colour hues: brown (128-40-40), red (216-40-40), and orange (216-128-40) were significant different (Figure 7).These colours are determined by type and amount of pigments in the scales (Figure 8 A-D). Nine spots (4, 5, 6, 8, 11, 17, A, B, and C) located on dorsal wing surfaces of *A. a. roeselia* presented variation, to this quantification were used 231 males and 385 females. In males and females, spots 4, 5, and 17, had frequencies of presence higher than absence (Table 1). Red spots D-G (Figure 4) presented no variation in both sexes. Size of males ranged from 24 to 30 mm (median = 27.0 mm; n = 149) and females from 25 to 34 mm (median = 28.0 mm; n = 129) being significant different between (Mann-Whitney U = 4241.0, $p < 0.0001$; Figure 9).

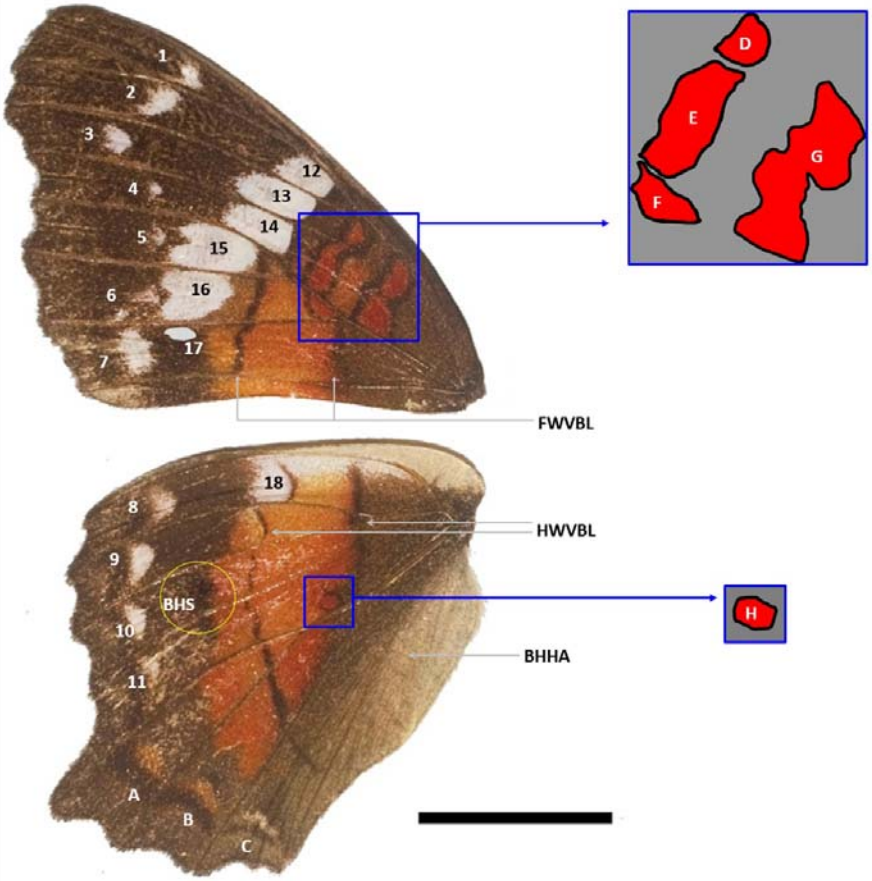



**Fig 4:** *Anartia amathea roeselia*. Spots and strips of colour pattern of wings showing the white spots 1-18, the red/orange spots A-C, the red spots D-G, on forewing and H on hindwing, the vertical black forewing (FWVBL) and hindwing (HWVBL) strips, and the black hindwing spot (BHS) that is diffuse and less visible on dorsal surface. Scale bar = 10 mm.

Spots and strips of colour pattern of wings showing the white spots 1-18, the red/orange spots A-C, the red spots D-G, on forewing and H on hindwing, the vertical black forewing (FWVBL) and hindwing (HWVBL) strips, and the black hindwing spot (BHS) that is diffuse and less visible on dorsal surface. Scale bar = 10 mm.

**Table 1:** Frequency (and respective percentages) of spot 4, 5, 6, 8, 11, 17, and spots A, B, and C on the wings of males (n = 231) and females (n = 385) of the butterfly *Anartia amathea roeselia* in in the study area during autumn 2010. Frequencies underlined are not significant differences from 1:1.

| | 4 | 5 | 6 | 17 | | 8 | 11 | A | B | C |
|---|---|---|---|---|---|---|---|---|---|---|
| **Males** | | | | | | | | | | |
| **Absent** | 33 (14.3) | 99 (42.9) | 101 (43.7) | 94 (59.3) | **White** | 19 (8.2) | 57 (24.7) | 5 (2.2) | 0 (0.9) | 4 (1.7) |
| **Present** | 198 (85.7) | 132 (57.1) | 130 (56.3) | 137 (40.7) | **Orange** | 212 (91.8) | 174 (75.3) | 226 (97.8) | 229 (99.1) | 227 (98.3) |
| **Total** | **231** | **231** | **231** | **231** | | **231** | **231** | **231** | **231** | **231** |
| **Females** | | | | | | | | | | |
| **Absent** | 71 (18.4) | 109 (28.3) | (0) | 185 (48.05) | **White** | 375 (97.4) | 68 (17.7) | 10 (2.6) | 5 (1.3) | 22 (5.7) |
| **Present** | 314 (81.6) | 276 (71.7) | 385 (100) | 200 (51.95) | **Orange** | 9 (2.6) | 317 (82.3) | 375 (97.4) | 380 (98.7) | 363 (94.3) |
| **Total** | **385** | **385** | **385** | **385** | | **385** | **385** | **385** | **385** | **385** |

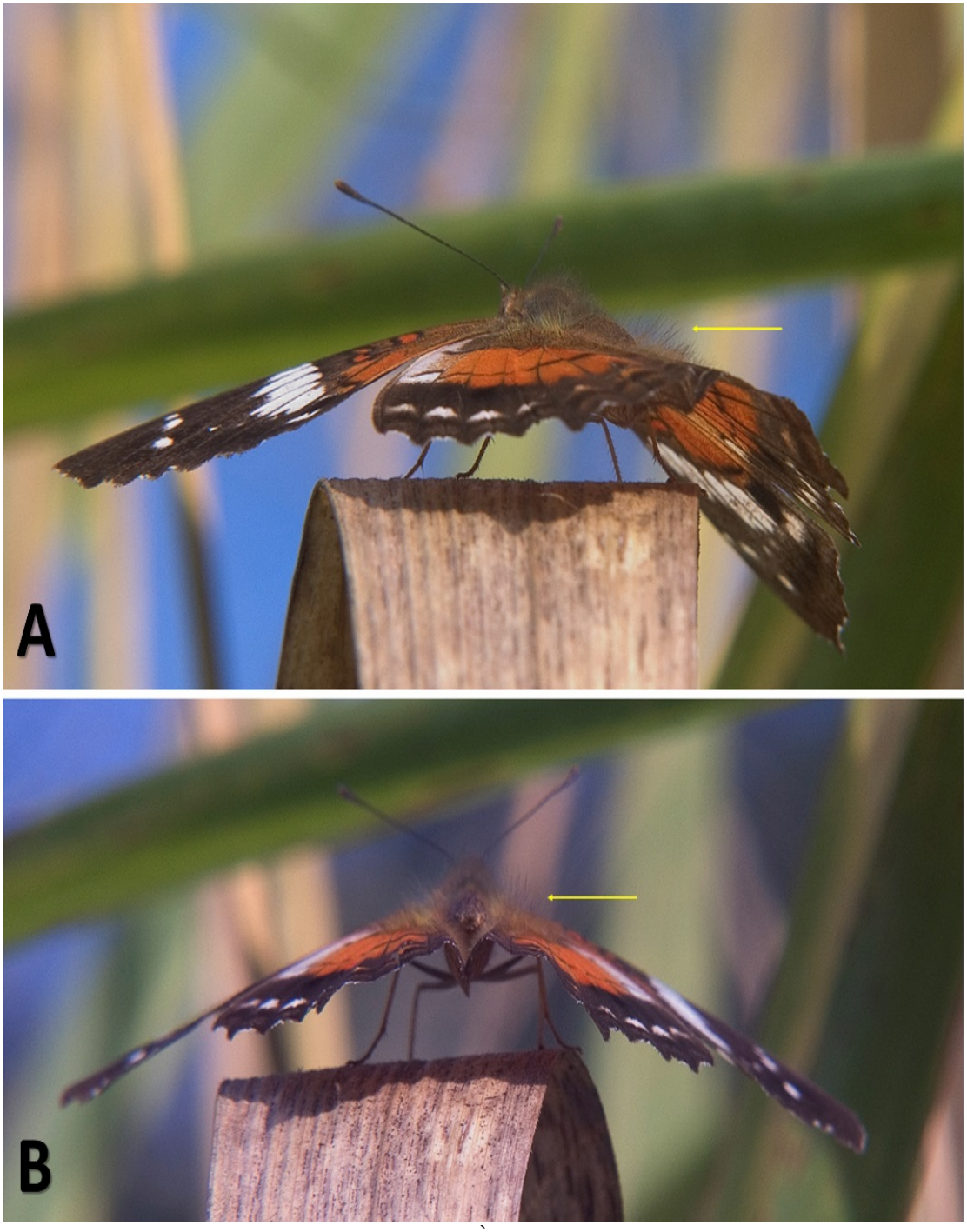



**Fig 5:** *Anartia amathea roeselia*. The yellow arrow indicates the hairs of the basal hindwing grey hair area exposed by a landed female. (A) Lateral view and (B) anal view. Photos, RBF June 2006.

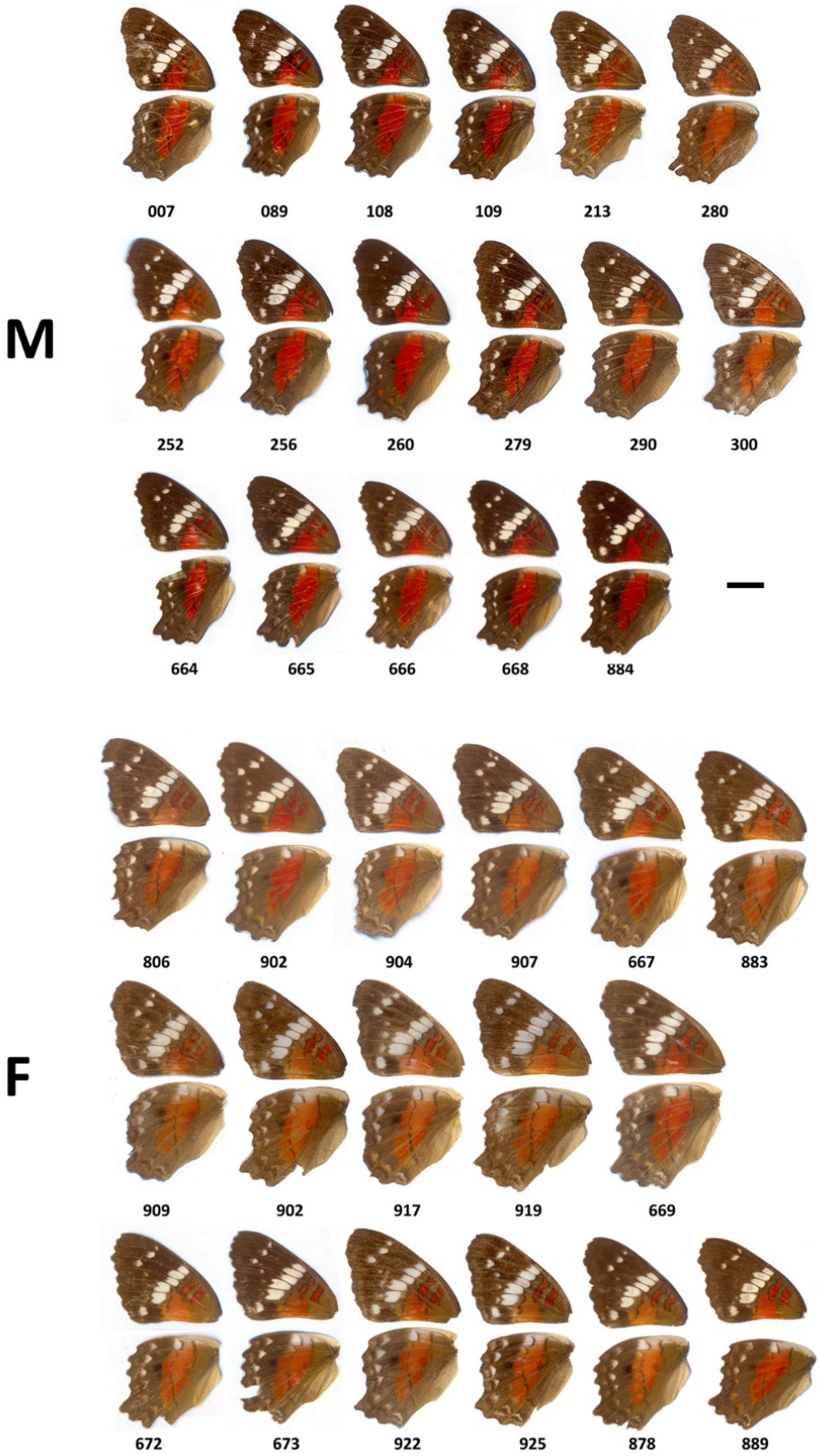



**Fig 6:** *Anartia amathea roeselia*. Variation of dorsal colour pattern of wings of males (M) and females (F) of samples. Numbers identify each sampled individual.

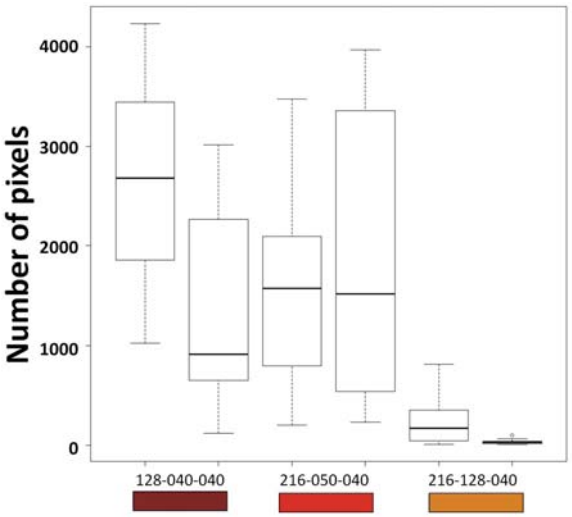



**Fig 7:** *Anartia amathea roeselia*. Number of pixels of the three most frequent colour classes: brown (128-40-40), red (216-40-40), and orange (216-128-40). Male data at left of each pair.

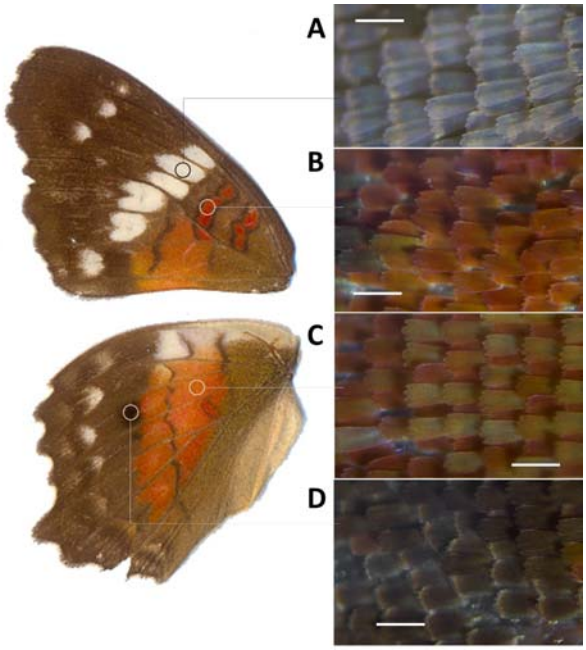



**Fig 8:** *Anartia amathea roeselia*. Details of scales at dorsal surface of wings. (A) White scales at white patch. (B) Red scales at red spot. (C) Red and orange scales at HROA. (D) Black scales at black spot. White bar = 0.1 mm.

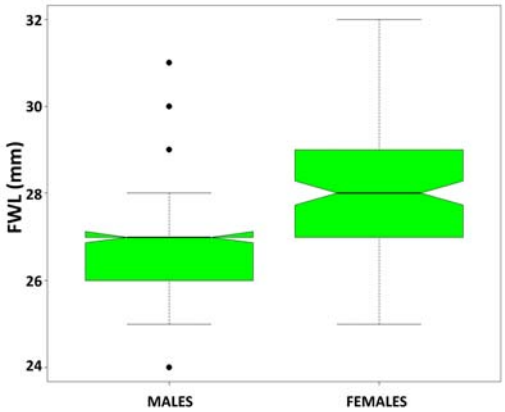



**Fig 9:** *Anartia amathea roeselia*. Forewing length (FWL) (mm) of both sexes in samples with females being bigger (Mann-Whitney U = 4241.0, $p < 0.0001$). Central horizontal line of the box is median; bottom and top edges of the box the first and third quartiles; length of the box equal to the interquartile range; circles lying beyond whiskers are outliers.

## 4. Discussion

The bigger size of females of *A. a. roeselia* was also found by Fosdick [17] and appears to be the common pattern found on other nymphalid species [27, 28, 29]. Indeed, Stillwell *et al.* [30] showed that 36 in 48 species of Lepidoptera (73%) have female-biased sexual size dimorphism although sexual dimorphism can be observed in a wide variety of other morphological traits [31].

The variation of red colour in both wings of butterflies of the same sex can be explained as a consequence of pigment degradation. Umebachi [32] determined that red pigments on the wings of *Vanessa*, a genus of the same subfamily of *Anartia*, is type C [33, 34], which is an ommatin, an ommochrome derived from the amino acid tryptophan. However, due to its chemical nature, the red rhodommatin [35] is degraded by continuous exposition to water and sunlight, especially short UV wavelengths, with colour fading with butterfly age. Females present the white spot 18 on SDA, which is not found on males. By other hand, males have FROA and HROA darker than females due to the presence of larger brown and orange tinted scales. However, the colour intensity of these red areas can became subdued with age. As choice of partner by females may involve the recognition of the colour pattern, old males could be in trouble to be accepted by females. The behaviour of these butterflies spreading their wings horizontally when landed permits that individuals of each sex signalize their gender status.

## 5. Conclusion

In spite of the observed variation, our results showed that the colour wing pattern of *A. a. roeselia* had enough differences to separate individuals of both sexes. Some of these traits are suitable to define the gender and other adequate to be used as morphological markers in population studies when the role of natural selection is been searched.

## 6. Acknowledgements

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## 7. Author contributions

RBF designed the sampling protocol, collected samples in the field, analysed data, and wrote the manuscript.
TSS collected samples in the field, and review the manuscript.
RRR analysed data, and review the manuscript.
MSC extracted traits from samples, and quantified sampled material.

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