

## Reproductive behavior in the annual fish *Austrolebias reicherti* Loureiro & García 2004 (Cyprinodontiformes: Rivulidae)

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Annual fishes inhabit temporary ponds that dry out seasonally and the adaptations to survive this extreme condition include high metabolic rates and an elaborate courtship behavior which ends in the deposition of drought-resistant eggs, capable of going through diapause stages in the substrate. The pronounced sexual dimorphism that these fishes show suggests that sexual selection could play a key role in the differentiation, speciation and evolution of this diverse group of fishes. However, there is scarce information about their reproductive isolation mechanisms, including detailed analysis of courtship signals. Herein, we analyzed, qualitatively and quantitatively, the reproductive behavior of *Austrolebias reicherti*. The behavioral units recognized in this study correspond with the previous analysis of other species of annual fishes. The most frequent unit in males was the lateral display where specific morphological and coloration patterns are displayed to the female. The female's high relative frequency and time of quietness suggest that this unit may have an evaluation role during courtship. In addition to visual displays during courtship, males perform vibrations of the dorsal and anal fins as well as body undulations; these indicate that mechanical signals may be important for attracting females. Our results support the hypothesis of multimodal signals. The conservation of behavioral patterns in courtship displays within *Austrolebias* suggests that species-level recognition and the barrier to hybridization may not occur at this stage, unless quantitative differences can be identified.

Os peixes anuais habitam charcos temporários que secam sazonalmente, e as adaptações para sobreviver a estas condições extremas incluem altas taxas metabólicas e um elaborado comportamento de cortejo, que culmina com a deposição de ovos resistentes à dessecação dentro do substrato, os quais são capazes de atravessar estádios de diapausa. O pronunciado dimorfismo sexual apresentado por estes peixes indica que a seleção sexual poderia estar exercendo um papel chave na diferenciação, especiação e evolução deste grupo diverso de peixes. Entretanto, informações acerca dos mecanismos de isolamento reprodutivo, incluindo uma análise detalhada dos sinais de cortejo, são escassas. Neste artigo é analisado o comportamento reprodutivo de *Austrolebias reicherti* de maneira qualitativa e quantitativa. As unidades comportamentais utilizadas neste estudo correspondem àquelas incluídas em análises anteriores de outras espécies de peixes anuais. A unidade comportamental mais frequentemente observada em machos foi a “exibição lateral”, onde características espécie-específicas da morfologia e colorido são exibidas para a fêmea. A alta proporção de ocorrência (em frequência e tempo) do comportamento de “quietude” nas fêmeas indica que o mesmo poderia desempenhar um papel avaliativo durante o cortejo. Outros sinais além dos visuais estão envolvidos no cortejo dos peixes anuais; os machos vibram as nadadeiras dorsal e anal e ondulam o corpo, o que indica que os sinais mecânicos poderiam ter influência na atração das fêmeas. Estes resultados conferem suporte à hipótese de sinais multimodais, recentemente proposta. Os movimentos de cortejo altamente conservados dentro de *Austrolebias* indicam que o reconhecimento específico e a barreira à hibridização poderiam não ocorrer nesta etapa, a menos que se encontrem diferenças quantitativas nestes sinais comportamentais.

**Key words:** Courtship, Killifish, Patos-Merín system, Quantitative behavioral analysis.

### Introduction

The family Rivulidae is one of the most specious taxa of the Neotropics, with several genera that show a unique annual

life cycle (Costa, 1998; Vari & Malabarba, 1998). Annual fishes inhabit temporary ponds that dry out seasonally and the adaptations to survive this extreme condition include high metabolic rates (Cardozo, 1999) and an elaborate courtship behav-

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ior (Vaz-Ferreira *et al.*, 1964, 1972; Belote & Costa, 2002, 2003, 2004) which culminates in the deposition of drought-resistant eggs in the substrate, where these eggs undergo diapause stages (Wourms, 1972). Another remarkable characteristic of many rivulids is their sexual dimorphism. In the genus *Austrolebias* Costa, 1998 (distributed along the Parana-La Plata basin and the Patos-Merín system) males of most species exhibit bright coloration on the opercular region and unpaired fins and a pattern of vertical bars or dots on body flanks; whereas females are brownish (with hues of yellow and/or green on the opercular region) with scattered dots (of diverse form) on body flanks and fins (Fig. 1). This pronounced dimorphism suggests that sexual selection could play a key role in the differentiation, speciation and evolution of this diverse group of fishes.

The *Austrolebias adloffii* species group (*sensu* Costa, 2006) is endemic to the Patos-Merín system. The group consists of at least five species, two of them having a parapatric distribution along the southwestern lowlands of laguna Merín (Loureiro & García, 2006); *A. reicherti* Loureiro & García 2004 to the north of arroyo Parao (rio Cebollatí basin) and *A. charrua* Costa & Cheffe 2001 to the south of this river. Furthermore, the area of contact is diffuse, and it has been proposed as a hybrid zone (García *et al.*, 2004; Loureiro & García, 2006).

Despite that these interesting evolutionary processes (*i.e.*, sexual selection and hybridization) may be acting in these species, there is scarce information about their reproductive isolation mechanisms, including detailed analysis of courtship signals. Belote & Costa (2004) qualitatively described the reproductive behavior of *A. charrua*; the general pattern of displays they observed corresponded to those previously reported for other species in the genus (Vaz-Ferreira & Sierra, 1964, 1972). However, no information is available for the parapatric *A. reicherti*. The goal of this study was to analyze, qualitatively and quantitatively, the reproductive behavior of this species.

### Materials and Methods

Fishes were obtained through field sampling in the type locality of the species (32°55'S, 53°54'W) using hand nets; specimens were transported to the laboratory where sexes were kept separately. Aquariums were kept at room temperature and constantly aerated; the fish were fed daily with live *Tubifex* sp.

Courtship behavior was observed in a special aquarium (33 x 15 x 25 cm) with the bottom completely covered with peat moss, 10 cm deep, resembling the natural substrate inhabited by annual fishes. Each trial consisted of one male and one female interacting for a period of 30 min, recorded with a Panasonic PV-DV 100 video camera. Each individual was tested only once and the total number of trials was 10.

Video recordings were analyzed with a Panasonic NV-HS 1000 video player. First, we defined the behavioral units of the courtship pattern. Subsequently, a complete sequence of these units was made simultaneously for males and females. This sequence was used to analyze the transition of behavioral units, and the frequency and duration of each unit in both sexes. From this data, flow diagrams for both sexes were constructed. Finally, the sequence was divided into three 10-min periods to analyze the time variation in the appearance of behavioral units in males. After the experiments, the fish were sacrificed with 2-phenoxyethanol and fixed in formalin (10%). Voucher specimens were deposited in the Vertebrates Collection of Facultad de Ciencias, Universidad de la República, Uruguay (Institutional code ZVC-P, Lot number 6965).

### Results

Eight behavioral units in males and five in females were identified. Units appearing only occasionally (one time) were removed from the analysis. The description of the most representative courtship displays of males and females with their relative frequency and duration time are shown in Table 1.

**Sequence of behavioral units.** Males exhibited a pale coloration when first introduced in the aquarium, with coloration intensifying when approaching a female. The reproductive behavior begins with the male alternating lateral displays (LD) with sigmoid displays (SD); when the female responds by swimming towards the male, two pathways may occur: the male may display an invitation to dive (ID) or an invitation to follow (IF). In the first case, if the female displays a diving acceptance (DA) the pair dives toward the substrate and mating (M) occurs. In the second case, the male may move to a different place in the aquarium and display another ID. If the female does not follow the male, then the male initiates a whole new sequence of displays (Fig. 2).

The flow diagram obtained for males *Austrolebias reicherti*

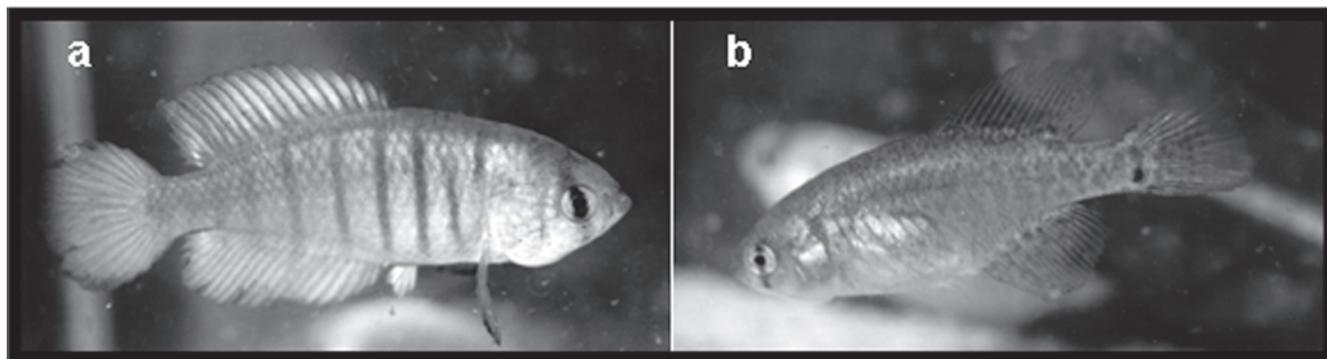
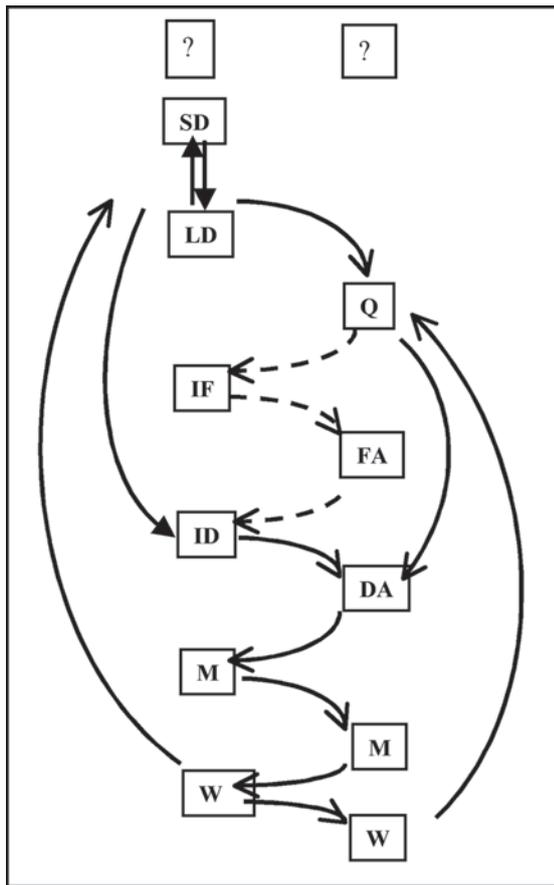
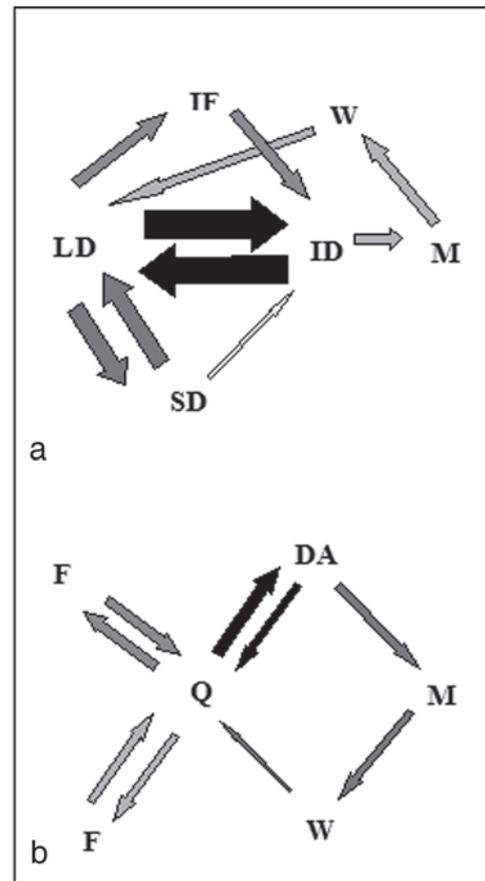


Fig. 1. Male (a) and female (b) of *Austrolebias reicherti*.



**Fig. 2.** Schematic diagram of behavioral units of sequence of courtship and mating in *Austrolebias reicherti*. Abbreviations of units shown in Table 1. Broken lines indicate alternative (less frequent) sequence.



**Fig. 3.** Flow diagram of behavioral units of transitions of males (a) and females (b) in *Austrolebias reicherti*. Thickness of arrows is proportional to frequency of transitions. Abbreviations of units shown in Table 1.

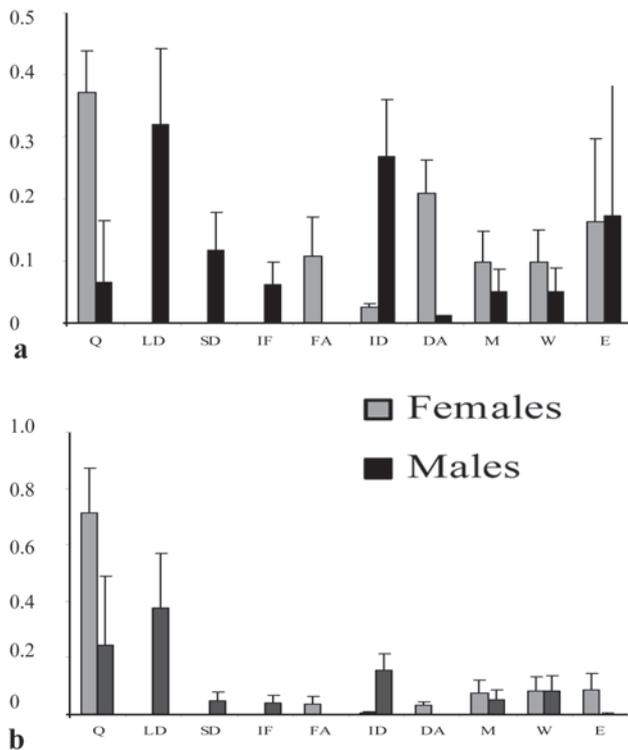
**Table 1.** Behavioral unit description, relative frequency and time of males and females of *Austrolebias reicherti*; SD = standard deviation.

Behavioral Units		Description	Relative frequency (mean ± SD)		Relative time (mean ± SD)	
			males	females	males	females
<b>Males</b>						
Lateral display	LD	Body still, dorsal and anal fins extended, which may vibrate or not	0.32 ± 0.12		0.38 ± 0.19	
Sigmoid display	SD	Lateral display with body undulations	0.12 ± 0.06		0.05 ± 0.03	
Invitation to follow	IF	Successive short swimming away and stops	0.06 ± 0.04		0.04 ± 0.03	
Invitation to dive	ID	Body in vertical or oblique position, with head over the substrate, and vibration of extended dorsal and anal fins	0.27 ± 0.09		0.15 ± 0.06	
Mating	M	Body placed by the side of female, pressing its body to the substrate. Unit performed from near the surface to completely buried	0.05 ± 0.04		0.05 ± 0.04	
<b>Females</b>						
Following Acceptance	FA	Female follows male (displaying IF)		0.11 ± 0.06		0.03 ± 0.03
Diving acceptance	DA	Females placed by the side of male touching its body		0.21 ± 0.05		0.03 ± 0.01
Mating	M	Female buried in the substrate (partially or completely) together with male		0.1 ± 0.05		0.07 ± 0.05
<b>Shared units</b>						
Quietness	Q	Body still	0.06 ± 0.10	0.37 ± 0.07	0.24 ± 0.24	0.71 ± 0.15
Wait	W	Individual remains still over the substrate after mating	0.05 ± 0.04	0.1 ± 0.05	0.08 ± 0.05	0.08 ± 0.05
Escape	E	Individual swims away from each other	0.17 ± 0.23	0.16 ± 0.13	0.001 ± 0.001	0.08 ± 0.06

(Fig. 3a) shows that the most frequent transition events was between ID and LD, followed by a shift from LD to SD; the transition between LD and IF was also observed. On the other hand, the most frequent behavioral circuits in females were those related to quietness (Q) and mating (M) (Fig. 3b).

**Quantitative Analysis.** The most frequent display of males was LD, followed by ID, SD and IF; whereas in females the most frequent display was Q, followed by diving acceptance (DA), following acceptance (FA), M, and wait (W) (Table 1; Fig. 4a). Concerning the relative time of displays in males, LD was the most common display, followed by Q, and ID; in females Q was the most common display, followed by W and M (Table 1; Fig. 4b).

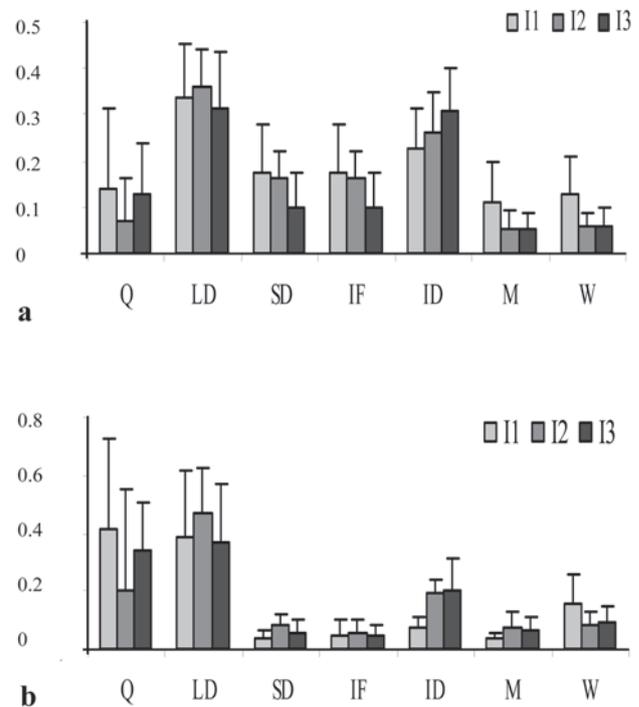
The following observations were made in relation to variation of frequency of units over time of experiment in males (Fig. 5a): Q was most frequent (circa 0.15) in the first and last intervals; LD showed a high frequency in all intervals, reaching the highest frequency in the second (0.35); SD was most frequent in the first and second interval with a frequency higher than 0.15, and the same occurred with IF; ID increased in relative frequency along the experiment, less than 0.25 in the first interval, to more than 0.25 in the second, and higher than 0.3 in the last one; M and W were higher in the first interval (0.1). In the analysis of relative time (Fig. 5b), the same pattern was observed, with the exception of M which increased in duration with time. Most displays were observed in the second interval.



**Fig. 4.** Relative frequency (a) and time (b) of behavioral units of males and females in *Austrolebias reicherti*. Abbreviations of units shown in Table 1.

## Discussion

The behavioral units (displays) defined in this study corresponded to the categories recognized in previous analyses of other species of annual fishes. Vaz-Ferreira *et al.* (1964) briefly described the reproductive behavior, without division of units, in *Austrolebias bellottii*, *A. nigripinnis*, *A. viarius*, *A. luteoflammulatus*, and *A. cheradophilus*. Several categories identified by those authors correspond to the units defined herein: dorsal and anal fin vibration in an extended position, fast and alternate movements, and repetitive action of substrate penetration. Vaz-Ferreira & Sierra (1972) defined display units in a social and reproductive context in *A. viarius*, *A. luteoflammulatus*, *A. bellottii*, and *A. nigripinnis*; many of which are equivalent to those defined in this study (lateral display, sigmoid display, invitation to follow, invitation to dive, mating, and wait). Recently, Belote & Costa (2004) analyzed the reproductive behavior of three species of *Austrolebias* (*A. cyaneus*, *A. nigripinnis* and *A. charrua*). These authors divided courtship behavior into five steps, each of them corresponding to one or more of the units described herein: Step 1 (**courtship displays**) includes lateral display, sigmoid display, invitation to follow; Step 2 (**invitation to submerge**) corresponds to invitation to dive; Step 3 (**submerging**) corresponds to diving acceptance; Step 4 (**spawning/fertilization**) corresponds to mating; and Step 5 (**emerging**) corresponds to wait. However, fine-scaled behavioral observations are always preferred in order to understand and discern specific



**Fig. 5.** Relative frequency (a) and time (b) of behavioral units of males in *Austrolebias reicherti* at 10-min time intervals. I1: first interval; I2: second interval; I3: third interval.

differences since it allows more precise quantification in comparative analyses. In this case, we showed that the units that compose the “courtship displays” of Belote & Costa (2004) have different frequencies and may appear in a different sequence, which justifies the adoption of a more detailed division of the behavioral units in the present study.

Reproductive behavior in species of *Austrolebias* is similar to that observed for species of related genera, e.g., *Simpsonichthys* (Belote & Costa, 2002). Differences in some stages of the reproductive behavior can be alternatively interpreted as specifically derived conditions or morphologic constraints. For example, many species with elongated unpaired fin rays, fold these fins in courtship behavior, which would be difficult to achieve in species with long fin bases and all fin rays of the same length (such as *Austrolebias*). Fin folding was observed in other Cynolebiatinae genera such as *Cynopoeilus*, *Leptolebias*, and *Campellolebias* (Vaz-Ferreira & Sierra, 1974; Costa *et al.*, 1988), in some species of *Simpsonichthys* (Belote & Costa, 2002), as well as in the distantly related annual fish species of the African Aplocheilidae, *Notobranchius guentheri* (Haas, 1976).

The high relative frequency and long duration of quietness and its high importance in the flow analysis in females indicates that it may have an evaluator role during courtship. Females of annual fishes carry few oocytes at a given time. Simpson (1979) indicated that females of *A. bellottii* lay between 8 and 12 oocytes each day, which may be interpreted as a limiting factor in the reproduction of these species. In this sense, female quietness may represent a way to assure species recognition and/or male fitness, thereby enhancing the chances of reproductive success. Both lines of evidence, together with sexual dimorphism, suggest that sexual selection is likely a strong evolutionary factor in the speciation processes of annual fishes, with female choice playing a key role in their evolution.

The high frequency and long duration of quietness in males, in the first 10 min of the experiment, may reflect an acclimation time for individuals to the test aquarium. In the following period is when a higher number of mating units occurred, decreasing in frequency during the third time interval. Lateral display was observed in higher frequency in the second interval while sigmoid display had the same frequency in the first and second intervals. However, both units lasted longer in the second interval; indicating that in the beginning the male may be investing energy in attracting the female. It is also interesting to note the apparent relation of invitation to dive and mating; while frequency and time of the first unit increases over time, the frequency of the second decreases and its duration increases. This could indicate a loss of female energy and motivation along the reproduction event. The male continues to try mating, while the female becomes less receptive, resulting in a decrease in mating.

Other signals besides vision seem to be involved in the courtship of annual fishes. As was observed in lateral display and invitation to dive, where males perform vibrations

of dorsal and anal fins, and in sigmoid display where the body undulates, mechanical signals seem to be very important in attracting females. Differences in fin morphology (i.e., dorsal fin base length) between species (Costa & Cheffe, 2001) and within species (D'Anatro & Loureiro, 2005) are common in *Austrolebias*. Do these differences in fin morphologies result in different signals when vibrating? No study has assessed this variation yet. Sound production by head movements has also recently been observed in one species of *Cynolebias* (Belote & Costa, 2003). Annual fishes have a highly developed cephalic lateral line system, suggesting the importance of extra-visual displays in this group of fishes. Furthermore, it would be interesting to explore the chemical signals that these fishes may be using. Previous reports showed that females *Xiphophorus pygmaeus* (Poeciliidae) prefer chemical signals from males of their own species than those from a sympatric species (*X. cortezi*; Hankison & Morris, 2003). These results support the hypothesis of backup signals proposed by Johnstone (1996), which indicates that multimodal signals allow a more precise evaluation of the sender condition (Candolin, 2003; Hebets & Papaj, 2005).

The high conservancy in courtship displays within *Austrolebias*, indicates that specific recognition and the barrier to hybridization may not occur at this stage, unless there are quantitative differences in courtship displays or in the aforementioned non-visual signals. High individual variability in frequency and duration of displays has been recorded by quantitative analysis. This kind of approach allows an objective and detailed comparison among species to elucidate the incidence of reproductive behavior in population differentiation, eventual speciation, and the establishment of reproductive barriers between lineages (Peretti & Córdoba-Aguilar, 2007). As mentioned before, *A. reicherti* is distributed parapatrically with *A. charrua* (Loureiro, 2004; Loureiro & García, 2006), with a zone of contact in arroyo Parao (río Cebollati basin). Unfortunately, quantitative analyses of *A. charrua* and other species of annual fish genera for comparative purposes are lacking.

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