Fatty acid methyl esters extracted from the cuticular surface of *Artemia franciscana* (Kellogs, 1906) (Crustacea: Anostraca) increase the swim speed of conspecific males

Ésteres metílicos de ácidos grasos extraídos de la superficie cuticular de *Artemia franciscana* (Kellogs, 1906) (Crustacea: Anostraca) incrementan la velocidad de nado de machos conespecificos

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ABSTRACT

Previous researches have established that the swim speed of some microcrustaceans is influenced by chemical compounds emitted by conspecifics. We examined the hypothesis that cuticular compounds present on the body surface of *A. franciscana*, the most widespread member of *Artemia* genus, play a role in the swim speed of conspecific males. The movements (swim) of one male confronted to a sponge soaked with female or male cuticular extract, were recorded during 30 minutes and the swim speed was determined using a behavioral tracking software (Ethovision 3.1, Noldus Technologies). As a control, the movements of one male confronted to a sponge soaked with salty water or with a mixture of the solvents used in the extraction (chloroform-methanol), was recorded. The results showed that cuticular compounds from either female or male increase 1.5 (ca.) times the swim speed of males in comparison with the controls treatments salty water and the solvents. There was no a significant difference between the controls (salty water and chloroform-methanol). Chemical characterization was developed by sterification of the cuticular extracts and analyses by GC-FID and GC-MS. Four saturated fatty acid (myristic acid, palmitic acid, estearic acid, arachidic acid) and five insaturated fatty acids (oleic acid, linoleic acid, linoleic acid 3n3, cis-11-eicosanoic acid, euric acid) were identified. Myristic acid was found in female cuticular extract, but not in male cuticular extract. Results suggest that chemical compounds present in the cuticular surface of *A. franciscana* females could have an important role in the intra-specific recognition in this specie.

Keywords: *Artemia*, swimming speed, cuticular compounds, fatty acids, chemical communication.

RESUMEN

Investigaciones anteriores han establecido que la velocidad de nado de algunos microcrustáceos se encuentra influenciada por compuestos químicos emitidos por conespecíficos. Nosotros examinamos la hipótesis de que los compuestos cuticulares presentes sobre la superficie corporal de *A. franciscana*, el miembro con mayor distribución mundial del genero *Artemia*, juega un rol en la velocidad de nado de machos conespecíficos. Los movimientos nado de un macho confrontado a una esponja empapada con extracto cuticular de una hembra o de un macho, fueron grabados durante 30 minutos y la velocidad de nado fue determinada usando un software de rastreo conductual (Ethovision 3.1, Noldus Technologies). Como control, fueron grabados los movimientos de un macho confrontado a una esponja empapada con agua de mar artificial o con una mezcla de los solventes usados en la extracción (cloroformo-metanol). Los resultados mostraron que los compuestos cuticulares tanto de hembras como de machos incrementan 1,5 veces (aproximadamente) la velocidad de nado de los machos, en comparación con los experimentos control donde se uso agua de mar artificial y los solventes de la extracción. No hubo diferencias significativas entre los controles (agua de mar artificial y cloroformo-metanol). La caracterización química fue desarrollada mediante esterificación de los extractos cuticulares y analizados por CG-DIL y CG-EM. Cuatro ácidos grasos saturados (ácido mirístico, ácido palmítico, ácido esteárico, ácido araquídico) y cinco ácidos grasos insaturados (ácido oleico, ácido linoleico, ácido linoleico 3n3, ácido cis-11-eicosanoico, ácido eurico) fueron

identificados. El ácido mirístico fue encontrado en los extractos cuticulares de hembras pero no en los extractos cuticulares de machos. Los resultados sugieren que los compuestos químicos presentes en la superficie cuticular de las hembras de *A. franciscana* podrían tener un importante rol en el reconocimiento intraespecífico de la especie.

Palabras clave: *Artemia*, velocidad de nado, compuestos cuticular, ácidos grasos, comunicación química.

INTRODUCTION

The brine shrimp *Artemia franciscana* is the most widespread member of *Artemia* genus, (Crustacea: Anostraca) and the only microcrustacean that inhabits hypersaline environments (Persoone & Sorgeloos 1980; Van Stappen 2002). It is an excellent extremophile model to understand the adaptation process both in nature and laboratory conditions, (Gajardo et al. 2002; Gajardo & Beardmore 2012), and a good resource for the aquaculture (Bengtson et al. 1991; Sorgeloos et al. 2001). *A. franciscana* have a sexual reproduction mode, and as other microcrustaceans that inhabiting large bodies of water, in relation to its size, the maintenance of the species depends on the probability of contact between males and females and of the subsequent effective mate recognition (Briethaupt & Thiel 2011). This encounter probability may be influenced by chemical factors (Briethaupt & Thiel 2011), factors that often influence directly on the swim speed (increasing the swim speed) of individuals (Seuront 2013). An increase of the swim speed will cover a larger area in less time (Gerritsen 1980; van Duren et al. 1998) and increases the likelihood of a male find and recognizes conspecifics (Gerritsen & Strickler 1977; Kiørboe 2008).

Several authors have established that one of the most effective ways for male crustaceans to find and recognize an opposite sex conspecific, is through sexual pheromones emitted by females (see Briethaupt & Thiel 2011). At the same time, the hypothesis that the swim speed in males of Crustaceans is modified by sex pheromone released by conspecific females has been tested by some authors, using as stimuli two main sources 1) alive females (van Leeuwen & Maly 1991; Tsuda & Miller 1998; Nihongi et al. 2004; Heuschele & Kiørboe 2012) and, 2) conditioned water from alive females (Yen et al. 2011; Seuront 2013). Tsuda & Miller (1998) showed that males of the marine microcrustacean *Calanus marshallae* (Copepoda: Calanoida) developed specific swim patterns (search and dance) in presence of newly molted females. These patterns imply an increase of the swim speed compared with the swim speed of males in absence of newly moulted females. In this way, the authors suggest that females release a sex pheromone which when received by the males they increase their swim speed.

Recently, Seuront (2013) reported the swim speed of males of another euryhaline Calanoid copepod, *Eurytemora affinis,* when was confronted to estuariane water, male conditioned water, non ovigerous female conditioned water and ovigerous conditioned water. Males increased significantly his swim speed in presence of non ovigerous and ovigerous females in contrast when were confronted to estuarine water, or male conditioned. The explanation proposed by the author was that the presences of a "diffuse background pheromone concentration" produced by females modify the swim behavior of males (Seuront 2013).

Has been reported that lipophilic compounds (glycoproteins, hydrocarbons and fatty acid) present on the body surface of decapod crustaceans (Caskey et al. 2009), and microcrustaceans (Snell & Morris 1993) play an important role in the mating behavior of these organisms (Caskey & Bauer 2005; Zhang et al. 2011; Kelly & Snell 1998; Ting et al. 2000; Ting & Snell 2003). However, there is no antecedent involving the presence of these compounds on the swim speed in crustaceans.

In this study, we examined the hypothesis that cuticular compounds present on the body surface of *A. franciscana* play a role in the swim speed of conspecific males.

MATERIAL AND METHODS

Sample Origin

A. franciscana individuals were collected in the Cejar Lagoon (3 ha in extent and an average water depth 10 m, and whose salinity at the time of collection was 200 ppt) in northern Chile (23°02'S – 68°13'W), and upon arrival at the Laboratorio de Química Ecológica of Universidad de La Frontera, they were placed in 5 L aquaria with water brought from the sample site, which was slowly replaced with artificial salty water (35 ppt). The juvenile instar meta-nauplii (2.2 - 2.6 mm in length) obtained from *Artemia* cultures (F3) were individually placed in Falcon tubes with 40 mL of artificial salty water (35 ppt) (approximately 20 days), to keep them virgin. Artemias were fed with *Dunaliella tertiolecta* (1.2×10^6 cells mL^{-1} per individual) every two days and constant aeration was provided by an aquarium pump (BOYU SC-7500) according to Gajardo & Beardmore (1993). All cultures were maintenance under 20±2 ºC and the water was replaced every two week.

Cuticular Extract Solution

Virgin organisms (approximately 20 days-old) were

grouped by sex and cuticular extraction from 40 virgin males (MCE) and 40 virgin females (FCE) was carried out. Cuticular components were extracted according to the methodology described by Caskey et al. (2009). Organisms were placed in a beaker with 5 mL of deionized water during 30 s in order to remove salt and other elements from the exoskeleton. Then, *Artemia* organisms were placed in a beaker with 5 mL of methanol (HPLC grade, Darmstadt - Germany) to make the surface miscible with the extraction solvent. Finally, *Artemia* organisms were removed and placed in a beaker with 12 mL of a mixed chloroform-methanol (2:1) (HPLC grade, Darmstadt - Germany) for 1 min to extract compounds from the surface. A volume of 450 microliter corresponds to 1.5 female or male equivalent. The extracts were filter through a syringe filter (Millipore, Durapore - 0.22 µm) with a syringe (BD Plastipak™ 0.50 x 15 mm) and stored to 4°C until bioassays and esterification.

Bioassay

In order to determine the effect of chemical stimuli o the swim speed of *A. franciscana* males, artificial sponges (3 x 5 mm) were used for depositing the cuticular extracts obtained from *A. franciscana* male or female. The sponges were previously washed with dichloromethane (HPLC grade, Darmstadt - Germany) in a soxhlet system and then rinsed with artificial salty water, in order to eliminate possible colorants and other compounds. Once dry, the sponges were soaked with cuticular extract solutions previously obtained.

Experiment consisted in placing one sponge in the center of a Petri dish (90 x 20 mm) on a flat base, above which a digital camera was placed for tracking and recorded the movement (swim speed). During 30 min 1 male was confronted to a sponge soaked with 450 µL of FCE or MCE. Sponge soaked with artificial salty water (SW) or a mixture of chloroform - methanol (2:1) (HPLC grade, Darmstadt - Germany) (CM) were used as controls. The entire tracks were analyzed using behavioral tracking software (Ethovision 3.1, Noldus Technologies) (Fig. 1). Each bioassay was repeated 10 times, under similar environmental laboratory conditions. The data were analyzed by ANOVA test to verify the a priori assumptions of normality and homoscedasticity. According to the above, differences between groups were tested

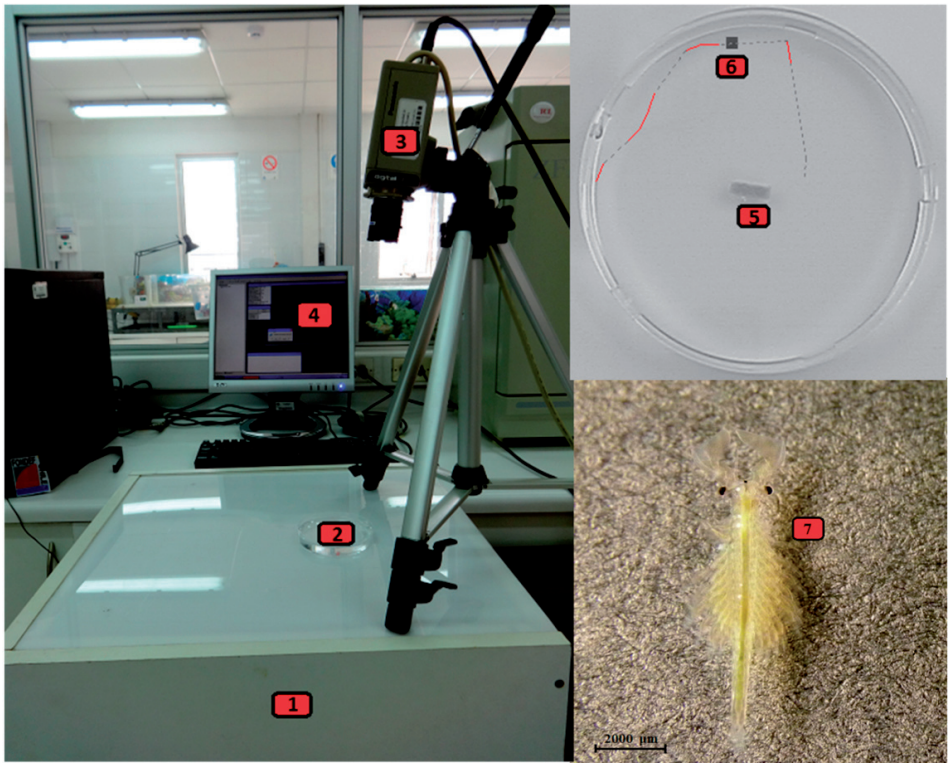

Figure 1. Bioassay set-up: (1) flat base, (2) Petri dish, (3) digital camera, (4) computer with behavioural tracking software (Ethovision 3.1, Noldus Technologies), (5) sponge (soaked with different extracts), (6) *Artemia* tracking movement during bioassays and, (7) *Artemia franciscana* male. N=

Figura 1. Sistema de bioensayo: (1) base plana, (2) placa Petri, (3) camara digital, (4) computador con el software de rastreo conductual (Ethovision 3.1, Noldus Technologies), (5) esponja (empapada con diferentes extractos), (6) rastreo del movimiento de *Artemia* durante el bioensayo y (7) un macho de *Artemia franciscana*.

by non-parametric Kruskal-Wallis test (P<0.05), using StatsDirect V.2.2 software (StatsDirect. Ltd., UK) followed by Conover-Inman's test for separation groups (P < 0.05) (Conover 1999).

Preparation of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAMEs) were obtained by adding 500 µL of an esterification reaction mix (methanol: hydrochloric acid: chloroform, 10:1:1) (HPLC grade, Darmstadt - Germany) to 3 mL of cuticular extracts. The mixture was stirred in a vortex for 20 s at 120 rpm, and was immediately incubated at 90 ºC for 45 min for completing the esterification reaction. The reaction tubes were left to cool at room temperature. Subsequently, 400 µL of distilled water was added to each tube and the mix was stirred. Then, FAMEs were extracted with hexane/chloroform (4:1 v/v, 3 x 400 µL). The samples were centrifuged to 3,000 rpm for 15 min. The organic fractions were pooled, and hexane (HPLC grade, Darmstadt - Germany) was added to obtain a final volume of 1.0 mL (Lewis et al. 2000).

Analysis and Quantification of the Fatty Acid Methyl Esters by GC-FID

Fatty acids were identified as methyl esters using gas chromatography-flame ionization detector (GC-FID) (Fison GC 8000 series model, Italy) equipped with a BPX70 capillary column (30 m x 0.22 mm x 0.25 µm film thickness). 1 µL sample, in splitless mode, was injected for each sample with the injector temperature at 250 ºC. The initial column temperature was 100 ºC for 1 min, increasing 6 ºC/min until reaching 250 ºC, and maintaining this temperature for 10 min. A commercial fatty acid methyl ester mix (Sigma Aldrich) was used for identification and quantification.

Analysis by GC-MS

FAMEs identification was corroborated by gas chromatograph (Model Focus; Thermo Electron, Waltham, MA) coupled to a mass spectrometer (model DSQ; Thermo Electron) (GC-MS) equipped with a DBP-5 capillary column (30 m x 0.22 mm x 0.25 µm film thickness). Helium was used as gas carrier at a flow rate of 1.5 mL/min. Ionization was by electron impact at 70 eV and 250 °C. The GC oven was programmed to remain at 40 °C for 1 min and then increased 5 °C/min to 250 °C. The identification was corroborated by means of the comparison between experimental mass spectra and those in the NIST mass spectral database (NIST ver. 2.0; Thermo).

RESULTS

Swim speed of *A. franciscana* male was significantly affected by the chemical stimulus applied on an acrylic sponge (Figure 2).

FCE (female cuticular extract) elicited a faster swim speed for male (2.897 cm/s ± 0.26) than the controls SW (salty water) (1.969 cm/s ± 0.24) (P = 0.0394) and CM (clorform-

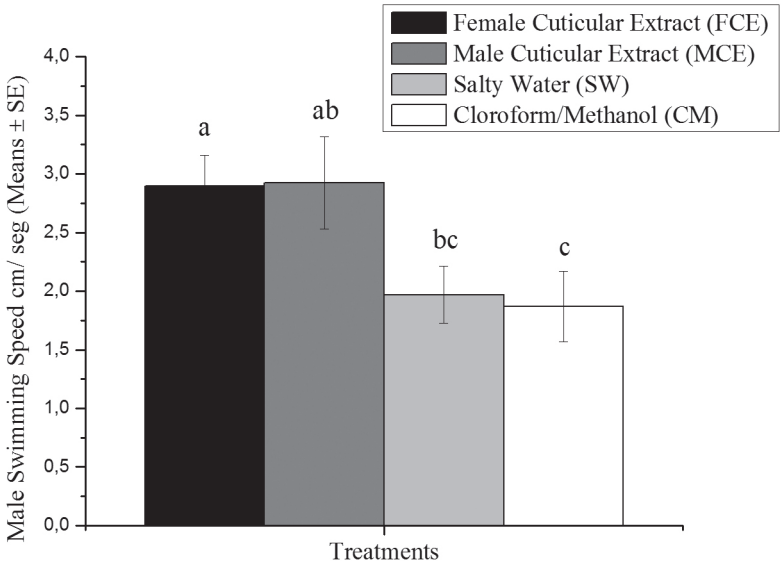

Figure 2. *Artemia franciscana* male swim speed elicited by a sponge soaked with female cuticular extract (FCE), male cuticular extract (MCE), salty water (SW) and, a mixture of chloroform: methanol (2:1) (CM) (N=10). Different letters indicate significant differences based on Kruskal-Wallis test followed by Conover-Inman's test (P < 0.05).

Figura 2. Velocidad de nado de *Artemia franciscana* macho suscitada por una esponja con extracto cuticular de hembra (FCE), extracto cuticular de macho (MCE), agua de mar artificial (SW) y una mezcla (2:1) de cloroformo: metanol (CM) (N=10). Diferentes letras indican diferencias significativas basadas en el test estadístico Kruskal-Wallis seguido por el test Conover-Inman (P < 0.05).

methanol) (1.868 ± 0.30) (P = 0.0176). On the contrary, MCE (male cuticular extract) elicited the same behavioural response that FCE (P = 0.743) and SW (P = 0.0791). There was no a significant difference between the controls (SW and CM) (P > 0.05) (Figure 2).

Chemical Analysis of Cuticular Extract

Qualitative differences were observed between fatty acid profiles of FCE and MCE from *A. franciscana* (Table I). Four saturated fatty acids (myristic acid, palmitic acid, estearic acid, and arachidic acid), and five unsaturated fatty acids (oleic acid, linoleic acid, linoleic acid, *cis*-11-eicosanoic acid, and euric acid) were identified by GC-MS analysis. FCE and MCE fatty acid profile shared the same compounds, with exception of the miristic acid, only present in FCE (4.6 µg/individual). Palmitic acid was the most abundant fatty acid found in both FCE (50.5 µg/individual) and MCE (57.0 µg/individual). By contrast, euric acid was found in small amount in both FCE (1.3 µg/individual) and MCE (1.1 µg/individual).

Table I. Identification by GC-MS of fatty acids from female cuticular extract and males cuticular extract of *Artemia franciscana.*

Tabla I. Identificación mediante CG-EM de ácidos grasos provenientes del extracto cuticular de hembras y machos de *Artemia franciscana*.

RT	Fatty acids	Area (%)		Individuals equivalent (mg/individuals)	
		Female	Male	Female	Male
12.30	Myristic acid	12.3	nd	4.6	nd*
14.98	Palmitic acid	41.1	42.9	50.5	57.0
17.48	Estearic acid	19.6	22.7	32.3	40.4
17.93	Oleic acid	11.7	10.2	31.5	37.7
18.74	Linoleic acid	1.9	1.3	6.0	5.4
19.66	Linoleic acid 3n3	6.0	3.3	18.2	24.5
19.81	Arachidic acid	0.7	2.3	11.7	2.8
20.22	*Cis*-11-eicosanoic acid	0.5	0.3	1.6	4.0
22.33	Euric acid	0.2	0.2	1.3	1.1

*nd: not determined.

DISCUSSION

There are antecedents suggesting that swimming speed, and other behavior related to the swimming as angular patterns and changes of direction, of several microcrustaceans is modified by chemical compounds emitted by conspecific (van Leeuwen & Maly 1991; Tsuda & Miller 1998; Nihongi et al. 2004; Yen et al. 2011; Heuschele & Kiørboe 2012; Seuront 2013). Here, we compared the effect of cuticular surface extracts of *A. franciscana* females or males on the swim speed of conspecific males. Results showed that males increase swim speed in the presence of both FCE and MCE suggesting two possibilities: 1) males compete between them, hence ability to move fast would be advantageous and 2) similar cuticular compounds could be produced by both males and females. The first possibility is relatively common in crustaceans, for example the aggressive behavior, that involves the use of the chelipeds against their opponents, exhibit by males of the shorecrabs *Carcinus maenas* (Sneddon et al. 1997) and by the hermit crab *Pagurus filholi* in the presence of mating partners (Okamura & Goshima 2010).Sneddon et al. (2003) reported such aggressive behavior in the shore crab *Carcinus maenas* males increased when exposed to conditioned water of conspecific females. In *Artemia* males, the ability to move faster has important benefits. Males forage microalgae or food particles, hence those more active are likely to get higher amount of food in a given period of time than those less active. Similarly, more active males can have advantage seeking mates, and when the mate partner is finally found. The more energetic males can have advantage, because could remain attached in amplexus to females (Mura and Gajardo 2011). Once a male grasp a female in amplexus by means of their modified antennas, females display energetic movements to dislodge males, hence those more active are likely to both remain attached to females (Zapata et al. 1990) and to ride for long time in the so-called riding position, thus favoring copula or, perhaps, preventing females to be available to other males (mate guarding) (Jormalainen 1998).

Our results indicate that both sexes produce similar cuticular compounds. The production of similar chemical compounds by both sexes (especially males imitating females) is known as chemical mimicry (Dettner & Liepert 1994). This term has been reported in conspecific interaction in flat lizards (*Platysaurus broadleyi*), garter snakes (*Thamnophis*

sirtalis parietalis), tropical ants (*Cardiocondyla obscurior*) and staphylinid beetle (*Aleochara curtula*) where males chemically mimic females to avoid aggression by other conspecific males (Peschke 1987; Cremer et al. 2002; Whiting et al. 2009; Shine et al. 2000). This ecological adaptation may be a tool to neutralize the aggressive or choosy pattern shown by females when males get them in amplexus, therefore this partial overlapping should be a way to identify suitable mates. However, this needs further investigation.

Fatty acids are commonly present in the cuticular extracts in insects (Blomquist et al. 1987) and less common in crustaceans (Caskey & Bauer 2005; Zhang et al. 2011). Caskey et al. (2009) reported the presence of three saturated fatty acids (palmitic acid, estearic acid, and arachidic acid) and two polyunsaturated fatty acids (eicosatetraenoic acid and eicosapentaenoic acid) in cuticular extracts of the caridean shrimp *Palaemonetes pugio*. Palmitic, estearic and eicosapentaenoic acids were present in all developmental stages of the *P. pugio* (postmolt parturial females, postmolt nonparturial females, postmolt males and intermolt females), but most frequently in the postmolt parturial females and, postmolt nonparturial females. Eicosatetraenoic acid was found only in postmolt parturial females and postmolt nonparturial females. Similarly, in the moth *Adoxophyes orana* (Lepidoptera: Tortricidae), whole body extracts obtained from males and females, showed that myristic and palmitic acid are male-specific, and could act as a key component of male pheromone inhibitory on the mating behavior of other conspecific males (Otter et al. 1989). The above is concordant with the differentiation of the fatty acid present in FCE and MCE. Myristic acid only was found in FCE, indicating that this fatty acid could have a sex-specific function. Admitting the complexity of the cues involved in the mate recognition of a biological system (Dicke & Takken 2006), the results of this work allows us to suggest that: 1) the similarity of the cuticular compounds between *A. franciscana* males and females could be have a role in the reducing male-male aggression and, 2) the delicate differentiation of this cuticular spectrum helps to choice potential mating partners.

We interpreted the increased swim speed of males in the presence of a FCE as the probable existence of a short-range or contact pheromone in line with other similar findings as the exposed by van Leeuwen & Maly (1991) and Nihongi et al. (2004).

Tapia et al. (2015) provides evidence of a highly specific mate choice between *Artemia franciscana* individuals collected from Cejar Lagoon in northern Chile (23°02'S–68°13'W): a courtship behavior and chemical cues implicated. Concordantly, cuticular extracts from males and females using in this work, provide evidence of the use of chemical cues in *A. franciscana.* Future work should evaluate (individually) the activity of each of the identified fatty acids and assess the proportions in which they are blended, as well as considering other components of the arthropod cuticle, such as hydrocarbons.

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