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SHORT COMMUNICATION

Regional differentiation of the cuticular surface structure in the mesoparasitic copepod *Cardiodectes shini* (Siphonostomatoida: Pennellidae) on a pygmy goby

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Abstract

Cardiodectes shini is a mesoparasitic copepod found on the heads of pygmy gobies. The copepod inserts its cephalothorax with antennary processes into the host tissues, while the trunk and egg sacs remain outside the host. The ultrastructure of the epicuticle surface differs among the antennary processes, cephalothorax, and trunk. In the antennary process, the epicuticle appears fuzzy and is less electron-dense than other parts of the body. This loose cuticle structure may be related to the absorption of nutrients in the host hemolymph. The cephalothorax and trunk have an electron-dense epicuticle: there is an array of minute protuberances on the epicuticle of the cephalothorax, whereas the trunk cuticle has no protuberances. This array of protuberances might be involved in suppression of the host immune response, because the cephalothorax has direct contact with the host connective tissues and similar structures are found on other parasitic copepods inhabiting host tissue.

Key Words: cuticle; fine structure; innate immunity; nipple array; parasitic copepod

Introduction

The body surface has many important roles as an interface between the inside and outside of the body. Therefore, the surface structure of the integument has functional significance. The features of the integument surface can differ among species depending on their habitat and behavior. Moreover, the features can differ among regions within an individual depending on the functions of each organ. It is possible that similar surface structures have multiple functions and play different roles in different species or different body parts. For example, submicron nipple arrays reduce light reflection on the compound eyes of moths by forming a refractive index gradient (Bernhard, 1967; Wilson and Hutley, 1982), and this anti-glare property probably decreases their visibility by predators. Similar nipple arrays have been found in various taxa of aquatic metazoans, such as annelids (Hausen, 2005), entoprocts (Nielsen and Jespersen, 1977; Iseto and Hirose, 2010), echinoderms (Holland, 1984), and tunicates (Hirose *et al*., 1997, 1999), although their functions are not well-understood. Using synthetic sheets bearing nanopillars that mimic a nipple array, we previously demonstrated the anti-glare property of nipple arrays under water (Hirose *et al*., 2015) and bubble repulsion on hydrophilic nipple arrays (Hirose *et al*., 2013).

Copepoda is one of the most diverse aquatic metazoan taxa and includes numerous parasitic species. According to Ho (2001), 3,521 copepod species have been reported from fish species, and more species are routinely being described. Nipple arrays have been found on the integumentary cuticles of some parasitic copepods that insert their body either partly or entirely into their hosts (Østergaard and Bresciani, 2000; Hirose and Uyeno, 2014). Since anti-reflection and bubble repulsion are not likely very important functions within the host body, the nipple arrays in these parasitic copepods may have other functions. On a synthetic multi-pillar surface mimicking a nipple array, the numbers of spreading and phagocytizing hemocytes per unit area are always smaller than those on a flat surface made of the same material (Ballarin *et al*., 2015), suggesting that the nipple array helps parasites suppress the anti-parasitic activities of host hemocytes such as encapsulation.

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Cardiodectes shini is a mesoparasitic copepod found on the heads of pygmy gobies: the cephalothorax of *C*. *shini* is embedded in the tissue between the host epidermis and the skull, while the trunk is exposed outside the host. Here, we compare the cuticular ultrastructure of the parts embedded in the host tissues and those outside the host.

Materials and Methods

A pygmy goby (*Eviota* sp.) was collected by SCUBA diving at a depth of about 30 m off Manza (Okinawa Island, Ryukyu Archipelago, Japan) on July 17th, 2013. A female *Cardiodectes shini* was attached to the host's head, with the anterior half of its body embedded in the connective tissue near the eyes. The host goby with the parasite was fixed in 2.5 % glutaraldehyde, 0.1 M cacodylate, and 0.45 M sucrose, and stored at 4 °C. The part of the host head including the copepod was excised with razor blades under a binocular stereomicroscope. The excised specimen was rinsed with 0.1 M cacodylate and 0.45 M sucrose and post-fixed for 1.5 h in 1 % osmium tetroxide and 0.1 M cacodylate. Then, the specimen was dehydrated through an ethanol series, cleared with *n*-butyl glycidyl ether, and embedded in Agar Low-viscosity Resin (Agar Scientific, England). Thick sections were stained with toluidine blue for light microscopy. Thin sections were stained with uranyl acetate and lead citrate and examined using transmission electron microscopy (JEM-1011, JEOL, Japan).

Results

Gross morphology

The body of *C. shini* consists of a cephalothorax with well-developed, branching antennary processes, a slender neck, and a bean-shaped trunk (Figs 1A, B). There was a pair of spiral egg sacs near the posterior end of the trunk. The *C*. *shini* cephalothorax was completely embedded in the host tissue, while the trunk was exposed entirely outside the host tissue and the neck situated in the transition zone between the inside and outside of the host tissue (Fig. 1B). The host tissue surrounding the antennary processes was reddish due to host erythrocytes, while the tissue around the cephalothorax was transparent (Fig. 1A).

In the resin-embedded specimen, the resin did not harden evenly in the internal tissues of the body of *C*. *shini*, causing large holes in the histological sections (Fig. 1C). The resin hardened well in the integumentary tissues and antennary processes, as well as in the host tissues, enabling us to examine these tissues histologically and ultrastructurally (see below).

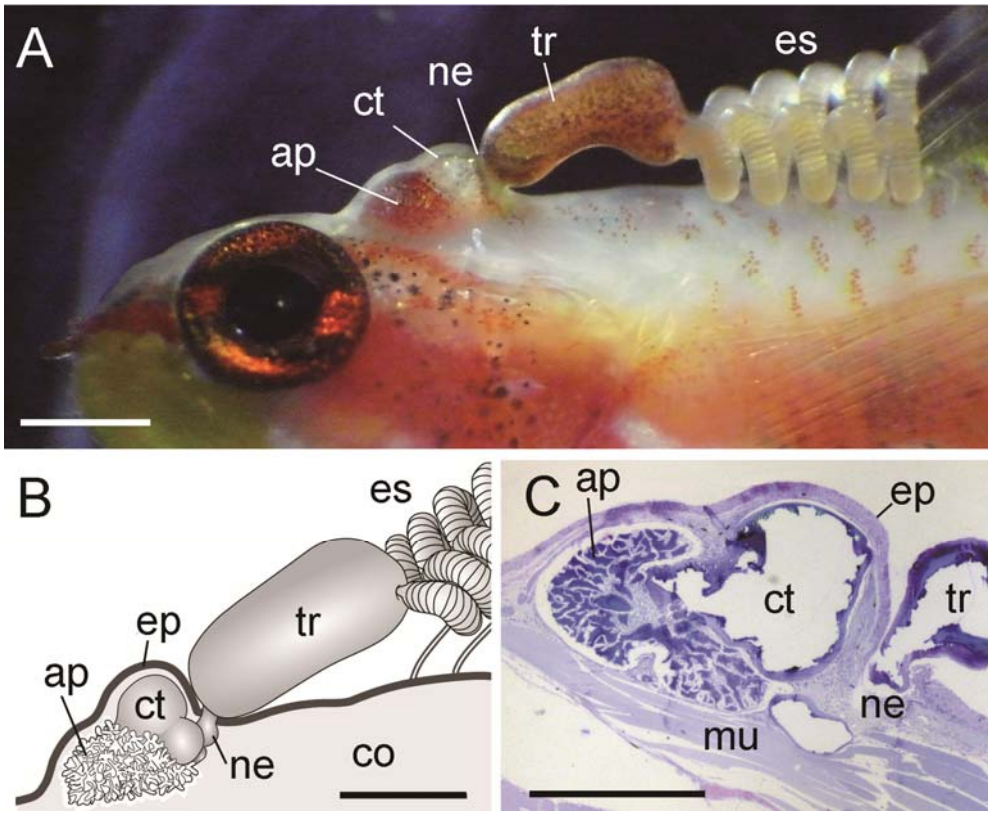

Fig. 1 *Cardiodectes shini* infesting the pygmy goby *Eviota* sp. (A) Live specimen. (B) Schematic drawing of *C*. *shini*. (C) Histological section stained with toluidine blue. ap, antennary processes; co, connective tissue of the host fish including muscle; ct, cephalothorax; ep, epidermis of the host fish; es, egg sac; mu, muscle of the host; ne, neck; tr, trunk. Scale bars: 1 mm (A, B), 50 µm (C).

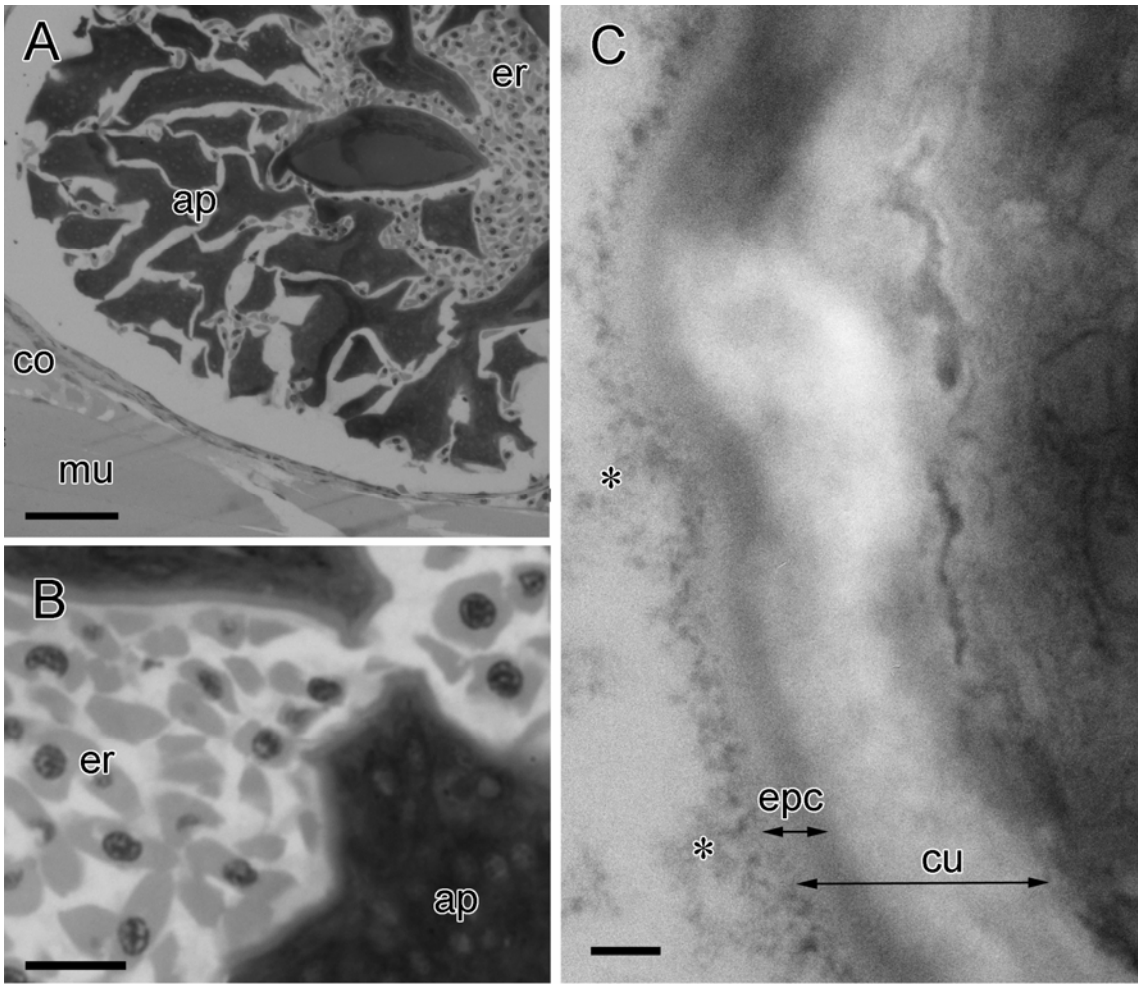

Fig. 2 Antennary processes of *Cardiodectes shini*. (A) Antennary processes (ap) in the host tissue (histological section stained with toluidine blue). (B) Enlargement of the processes and host erythrocytes (er) in the space among the processes (histological section). (C) Cuticle (cu) and epicuticle (epc) of the antennary processes (TEM). Asterisks indicate fibrous material on the cuticular surface. co, connective tissue of the host fish; mu, muscle of the host fish. Scale bars: 50 µm (A), 10 µm (B), 200 nm (C).

Antennary process

The antennary process is a nodular organ that originates from part of the antenna, which is the second appendage on the cephalothorax. In the histological sections, the antennary process was observed as an aggregate of sections of the branching processes that were heavily stained with toluidine blue (Fig. 2A). It was located in a sinus enclosed by the host connective tissues; there was a considerable gap between the host tissue wall and antennary processes. There were many host erythrocytes in the spaces among the branching processes, whereas no host hemocytes adhered to or encapsulated the processes. In the electron micrographs, the antennary process was comprised of electron-dense cells that were covered with a moderately electron-dense cuticle (Fig. 2B). The cuticular layer was about 1 µm thick and included a 200-nm-thick epicuticle. The surface of the epicuticle appeared fuzzy, and much fibrous material was seen (Fig. 2C).

Cephalothorax

The cephalothorax was spherical and embedded entirely in the host tissue. Since the resin hardened poorly in the inner cephalothorax tissues, we could only examine the integumentary tissues, *i.e.*, the cuticular layer and epidermis, in the histological and ultrathin sections. The cuticular layer was at least 5 µm thick and the surface was often directly in contact with the host connective tissues (Figs 3A, B).

The cuticle consisted of a fibrous matrix, and an electron-dense 10 - 20-nm-thick epicuticle formed the outermost layer of the cuticle (Figs 3C, D). Some electron-dense material was found in the cuticular layer beneath the epicuticle. Electron microscopy showed a gap between the cuticle and host tissue, and no host cells adhered to the cuticular surface. Many minute protuberances were crowded on the epicuticle; each protuberance was about 50 nm in height and 40 nm in diameter at the base, and the protuberances appeared to form a nipple array

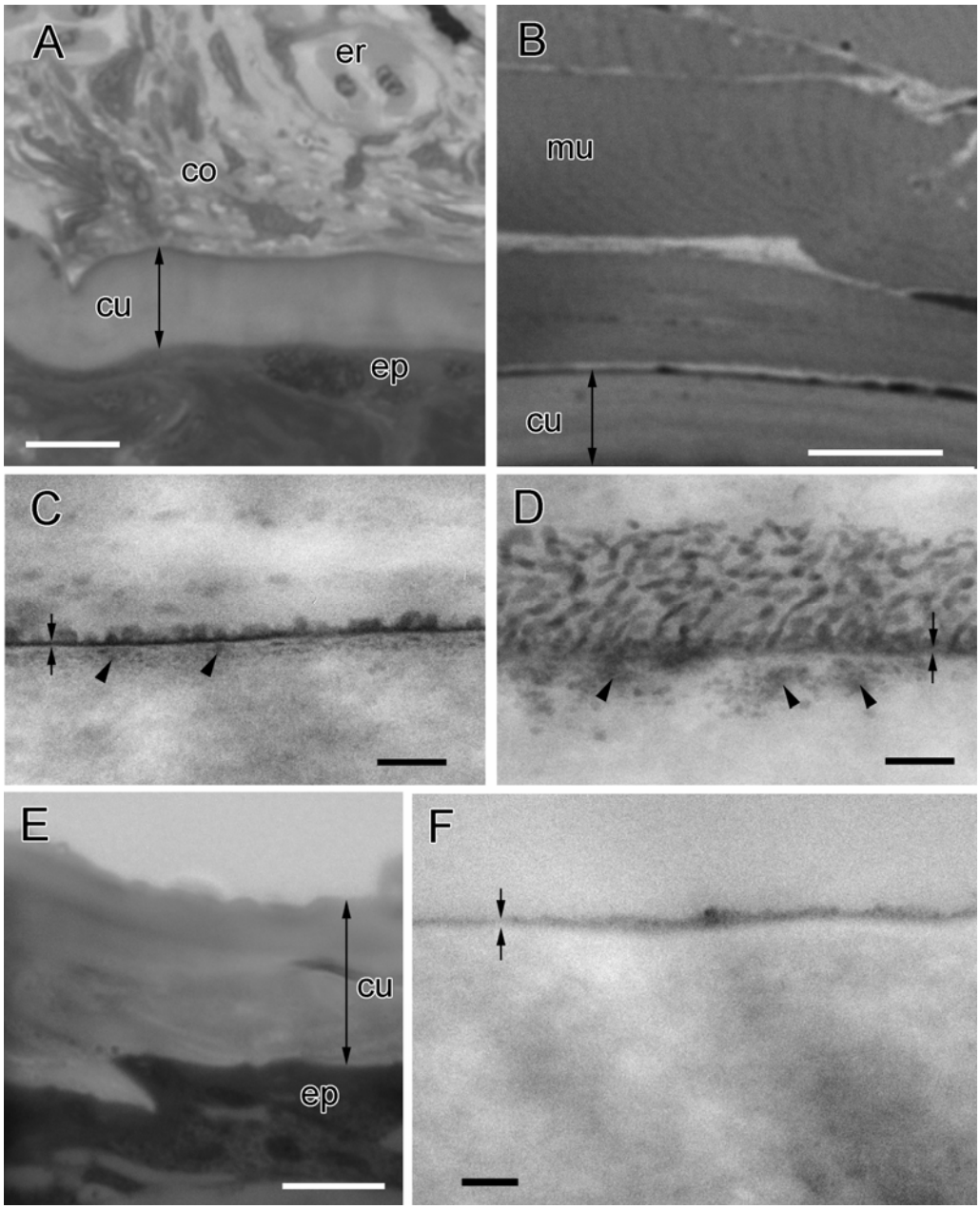

Fig. 3 Cuticular layer of the cephalothorax (A-D) and trunk (E, F) of *Cardiodectes shini*. A, B, and E are histological sections stained with toluidine blue, and C, D, and F are transmission electron micrographs. Arrowheads indicate electron-dense materials beneath the epicuticle. Facing arrows indicate the epicuticle layer. co, connective tissue; cu, cuticle; ep, epidermis of *C*. *shini*; er, erythrocytes; mu, muscle. Scale bars: 10 µm (A, B, E), 200 nm (C, D, F).

(Fig.3C). In some areas on the cephalothorax, the protuberances were markedly elongated, and an array of long protuberances formed a 300-nm-thick layer over the epicuticle (Fig. 3D).

Trunk

The trunk was exposed outside the host tissue and was connected with the posterior parts of the cephalothorax via the neck. The cuticular layer was at least 15 µm thick (Fig. 3E), while the epicuticle was about 30 nm thick and had no minute protuberances (Fig. 3F).

Discussion

C. shini is a mesoparasitic copepod that inserts its cephalothorax into the connective tissue of the head of the host fish. Our observations revealed that the cuticular layer of *C*. *shini* differs in thickness and surface structures among parts of the body. The cuticular layer was ~1 µm thick on the antennary processes, ~5 µm thick on the cephalothorax, and ~15 µm thick on the trunk. In parasitic copepods, the cuticle is generally very thin and the integument is often suggested to be a site of nutrient uptake

especially in the species lacking alimentary tract (reviewed in Bresciani, 1986). Some copepods have microvilli-like projections (*i.e*., microvillosities) on the epicuticle that are supposed to function in the nutrient absorption in the host tissue (Bresciani, 1986). For instance, in a pennellid copepod *Phrixocephalus cincinnatus* Wilson, 1908 that infects the eyes of flatfishes, the cuticle surface of the holdfast is covered with numerous, fine microvillosities that are occasionally in contact with host cells and may be involved in molecular exchange between parasite and host (Perkins, 1994). If the antennary process of *C. shini* is a nutrient-absorbing organ, branching of the nodular processes might have been an adaptation to increase the surface area, and the copepod is thought to absorb nutrients in the host hemolymph via the cuticle layer. Therefore, it is reasonable to believe that the processes have a thin cuticle, and the electron density of the epicuticle is less than on other parts of the copepod body because the cuticular layer of this organ should be permeable to soluble nutrients in the hemolymph. In comparison, the cuticle must be robust to maintain and protect the body elsewhere, especially the exposed trunk. A congener, *Cardiodectes medusaeus* (Wilson, 1908), invades the heart of lantern fishes, and the cuticle of frontal processes constituting the attachment organ is about 1 μm in thickness and covered with microvillosities (Figs 8 - 10 in Perkins, 1985). This species is supposed to absorb small molecules via the thin cuticle of the attachment organ in addition to the oral ingestion, considering the structural similarity with other specialized endoparasites (Perkins, 1985). The fuzzy, fibrous material on the cuticle surface of *C*. *shini* may correspond to the microvillosities (asterisks in Fig. 2C). The presence of the mouth tube suggests that *C*. *shini* feeds on the host's blood via its mouth as in the congener, *C. medusaeus* (see Uyeno, 2013). Although *Parachordeumium amphiurae* (Hérouard, 1906), an endoparasitic copepod on brittle stars, has a functional digestive system, it has been suggested that it supplements ingestion by absorption through the epidermis (Østergaard, 1998).

On the outermost surface of the cuticle on the cephalothorax, there is an array of cuticular protuberances (nipple array) where the host tissues are in close contact with the copepod body. By contrast, these structures were not found on the cuticular surface of the trunk, which is located outside the host tissues, or antennary processes, which are located in a connective tissue sinus in the host. In other words, the nipple array was found on the cuticle that would interact closely with the host connective tissues. Therefore, the presence or absence of cuticular protuberances is a clue to understanding the function(s) of the nipple array in *C*. *shini*. Nipple arrays on the cuticular surface have been found in some parasitic copepods, including *Ophioika* sp. infesting a brittle star (Østergaard and Bresciani, 2000) and *Mihbaicola sakamakii* infesting the branchiostegal membranes of groupers (Hirose and Uyeno, 2014). The body of *M*. *sakamakii* is located in a pouch composed of thickened host epidermis; consequently, the copepod body is located outside the host tissue topologically. The protuberances are 20 - 40 nm high in *Ophioika* sp., about 60 nm high in *M*. *sakamakii*, and about 50 nm high in *C*. *shini* (Fig. 3C), although the protuberances in *C*. *shini* are sometimes elongated and form a 300-nm-thick layer (Fig. 3D).

Host organisms usually have an innate immune system to eliminate foreign organisms within their body. Since endo- and mesoparasitic organisms are usually exposed to host tissues, they need to have a mechanism to resist or reduce host attacks. A nipple array might reduce cellular responses such as phagocytosis and encapsulation, because the cell-spreading and phagocytic activities of hemocytes are suppressed on synthetic nanopillar-covered sheets mimicking a nipple array (Nomura *et al*., 2005; Ballarin *et al*., 2014). Copepod parasitism leads to local inflammation (Dezfuli *et al*., 2003), but the nano-scale nipple array on the body surface might reduce the adherence and spread of host immunocytes. A nipple array might also reduce surface friction because synthetic micro-dimple arrays are a low-friction surface (Hirai *et al*., 2013). For parasites embedded in host tissue, a low-friction surface would help retain some motility and allow tissue/cells around the body to detach. In *C*. *shini*, it is not clear whether the 300-nm-thick layer formed by the elongated protuberances has the same functions as a nipple array. The layer does form considerable space between the host tissue and body surface, which may reduce host attacks. The trunk cuticle of *C*. *shini* has no cuticular protuberances, probably because the function(s) of the nipple array is not necessary on the trunk, which is located outside the host and has no contact with host tissue. Although a nipple array was not found on the epicuticle of the antennary processes, a dense epicuticle with protuberances might be unsuitable for a nutrient-absorbing organ. It is not known why the host tissues are not in close contact with the processes and no host hemocytes adhere on the cuticular surface. Another mechanism may prevent host fish immune responses.

In parasitic copepods, the presence of a cuticular nipple array is thought to be an adaptation to enhance resistance to host defensive responses. Accordingly, similar structures are expected to be found on the cuticles of many other parasitic copepods inhabiting host tissues. To test this idea, a further survey of the cuticular ultrastructure of endo- and mesoparasitic copepods is necessary.

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