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Epidermis structure and blood parameter differences between sculpin *Cottus gobio* and Siberian sculpin *Cottus poecilopus* from the Morava watershed

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Abstract. The epidermis structure of the studied specimens of *Cottus gobio* and *C. poecilopus* from the Morava watershed showed important differences. We noted a lower number of sacciform secretory cells in *C. gobio*, and also differences in relation to reproductive activity (the decrease in the number of secretory cells during the spawning period in both species). Significant differences were found in the erythrocyte count ($1.90 \pm 0.15 \text{ T.l}^{-1}$ in *C. gobio* and $1.57 \pm 0.07 \text{ T.l}^{-1}$ in *C. poecilopus*), whereas the leucocyte count did not differ. A three-day-long exposure in higher temperature and lower oxygen saturated water conditions caused a marked increase in both blood parameters. These differences can be related to the distant physiological and ethological requirements of the species.

Key words: goblet cell, sacciform cell, erythrocyte, leucocyte

Introduction

Sculpin *Cottus gobio* Linnaeus, 1758 and Siberian sculpin *Cottus poecilopus* Heckel, 1837 are classified among freshwater representatives of the Cottoidei suborder, which inhabits mountain streams in Europe. The distribution range of both species might partially overlap, but the appearance of both species in the same localities is only occasional (*C. poecilopus* usually reaches higher elevations than *C. gobio*) (Kottelat & Freyhof 2007). In such cases the possibility of hybridization is mentioned (Nyman & Westin 1968, Andreasson 1969, Čihař 1969). Both species are differing only in several traits (namely black striped vs. white ventral fins) and slightly greater size in *C. poecilopus* (Baruš & Oliva 1995).

There exist several parameters, whose variability among taxonomically related fishes may help to study their physio-morphological differences. One of the most important is a number of blood cells, especially

the erythrocytes, whose count can constitute a dominant factor in habitat preference of particular species with respect to the oxygen saturation (Baruš & Oliva 1995). The morphology represents another important feature. Although fish epidermis is apparently simple, it can be considered as a dynamic organ expressing great variability among taxonomical and ethological groups, especially in the case of thickness, in the number of secretory cells of different categories and in the occurrence of melanophores (Whitaker 1986, Knoz & Halačka 1991).

In general, the fish can be characterized by the ability of a cutaneous respiration (Jakubowski 1960, 1963). Hence the epidermis thickness, as a barrier between water environment and blood vessels in dermis, can constitute the limiting factor for gas exchange across the skin.

Most often, there are three types of epidermal cells except epidermocytes – goblet, club and sacciform

(Whitear 1986). The goblet cells are located in the epidermis of the majority of fish; their secretion has a glycoproteinous character. During their development, these cells move from the base to the surface of the epidermis, where they break up and release their secretion. This process facilitates the permanent renewal of fish mucus. The main function of this mucous film is to ensure the essential physical-mechanical features of the body surface and its barriers against infection (Whitear 1986, Knoz & Halačka 1991).

Club cells, termed also “alarm substrate cells” or “Schreckstoffzellen”, are present in the superorder Ostariophysi (e.g. Cypriniformes and Siluriformes) and Gonorynchiformes (Pfeiffer 1977, Whitear 1986). They are usually located in the middle of the epidermis and do not communicate with the epidermal surface. Their substances are released into the water following their rupture, especially after injury by predators, and these evoke a relevant “fright reaction” in fish nearby (Frisch 1941, Pollock et al. 2005). The reduction of the number of club cells in the epidermis in some fish species during the spawning period is explained by false alarm elimination in connection with non-predatory caused injuries in the spawning area. In some cases, this reduction is connected with a partial reduction in the number of goblet cells (Smith & Smith 1983, Knoz & Halačka 1991). According to new findings (Chivers et al. 2007, James et al. 2009), club cells may also provide an immune reaction against ubiquitous environmental antigens.

Sacciform cells are characteristic for Perciformes and Gadiformes, though they can occur exceptionally in Salmonidae (Pickering & Macey 1977, Whitear 1986). The exact function of sacciform cells is still unclear; their proteinous secretions show highly variable chemical characteristics. The alarm reaction activation by skin extract application in some sculpins such as *C. cognatus* Richardson, 1836 (Bryer et al. 2001, Chivers et al. 2001) and *C. perplexus* Gilbert et Evermann, 1894 (Chivers et al. 2000) confirms that they are also able to produce alarm substances analogous to those of the club cells.

This study focuses on main physio-morphological differences between both species, especially in the quantity of erythrocytes and leucocytes in optimal conditions and also under stress by hypoxia. Another aim was to evaluate the differences in the morphology of epidermis (e.g. mainly its thickness and the presence and volume of the particular secretory cells) between species (*C. gobio* vs. *C. poecilopus*), gender (male vs. female) and reproductive phases (spawning vs. non-spawning).

Material and Methods

Study area

In the Morava watershed mainly *C. gobio* is presented. One of the exceptions is a location near the town of Jimramov, where the occurrence of *C. poecilopus* has been recorded, probably due to its unintended importation with brown trout *Salmo trutta* Linnaeus, 1758 fish stock during last century (Lusk 1993). Its occurrence is limited to only two Svatka River tributaries, the Trhonický brook and the River Fryšávka near Jimramov. Solitary *C. poecilopus* individuals are rarely recorded in the River Svatka, usually in the stretch between the confluences of each tributary.

The studied individuals (see Table 1) were captured in the River Svatka at Jimramov (49°36' N, 16°13' E) (*C. gobio*) and in the Trhonický brook (*C. poecilopus*), about 500 meters upstream from its confluence with the River Svatka at the town. The River Svatka reaches a width of 13-16 m in the mentioned locality; the riverbed is stony, partially covered with sand and small pebbles; the river-basin slope is 1 : 500. The Trhonický brook is about 1-2 m in width; the rocky riverbed is covered by stones; the slope is near to 1 : 10. The basic parameters of both streams are summarized in Table 2.

Table 1. The studied *C. gobio* and *C. poecilopus* males (M), females (F) and juveniles (J).

	<i>Cottus gobio</i>			<i>Cottus poecilopus</i>		
	M	F	J	M	F	J
May (spawning period)	6	17		17	6	
July-October	18	13	3	14	25	6

Erythrocyte and leucocyte blood count

In twelve individuals of *C. gobio* and in the same number of *C. poecilopus* specimens, captured in May, a blood sample was taken from the caudal vein to assess the numbers of erythrocytes and leucocytes (Svobodová et al. 1991, Vetešník et al. 2006). To monitor the reaction to deterioration of the environmental conditions, another six individuals of each species were placed into the tank for a 72-hour-long period, after which blood samples were taken by the same procedure. The River Svatka environment during the capture was characterized by a temperature of 9.2 °C and an oxygen saturation of 10.5 mg.l⁻¹ (98.5 %); in the Trhonický brook, the temperature was 8.7 °C and the oxygen saturation 10.7 mg.l⁻¹ (98.5 %). In the experimental tank the temperature was maintained at 18.0 °C and oxygen saturation at 7.3 mg.l⁻¹ (79.9 %).

Table 2. Main characteristics of the sampling sites during the vegetation season of 2009.

	Depth (m)	Flow velocity (m/s)	Discharge (m ³ /s)	Oxygen saturation (%)	pH	Water temperature (°C)	Water conductivity (mS/m)
The River Svratka	0.35-0.41	0.38-0.48	2.4-2.6	96.0-101.6	6.8-7.6	7.7-15.6	13.0-17.4
Trhonický brook	0.08-0.12	0.28-0.40	0.04-0.10	99.1-105.6	7.7	8.7-15.5	18.2-24.6

Obtained data were statistically processed as a small sample analysis (Horn 1983) using software QC-Expert 2.5 (TriloByte Ltd., Czech Republic). Differences between species (*C. gobio* vs. *C. poecilopus*) and treatment (field conditions vs. stressed by hypoxia) were analysed by means of a two-way analysis of variance (ANOVA) using software NCSS 2007 (NCSS, LLC.)

Morphology of the epidermis

Fish were euthanized with an overdose of anaesthetic (2-phenoxyethanol); the standard length (SL) was measured and the gender macroscopically determined. Skin samples of approximately 5 × 5 mm were taken from the dorsal part of the head, fixed in Bouin solution for 2-3 days and transferred to 70 % ethanol for storage. According to the standard method, the samples were embedded in paraffin and cut into slices across the epidermis. Staining according to Mallory and Papanicolaou was subsequently applied. The assessed parameters were absolute (A) and relative (A/SL) thickness of epidermis and the relative rate of the secretory cells in epidermis volume (B) using a morphometric point network of density of 10 µm. Obtained values were transformed to the absolute secretory cell volume of the epidermis (A × B/100; Halačka et al. 1991, Halačka et al. 2010). For more accurate specification of the glycoprotein secretion and subsequent identification of the type of secretory cells, histochemical staining using Alcian blue at pH 2.5 (acid glycoproteins) or 1.0 (only sulphated acid glycoproteins) (Kiernan 1981) and PAS (neutral and acid sialated glycoproteins) (Horobin & Kevill-Davies 1971) were used, including a combination of both most often applied in studies focused on fish epidermis (e.g. Pickering & Macey 1977, Zaccone 1980). The classification of epidermal cells was undertaken according to Whitear (1986).

To describe the relationship among groups (*C. gobio*, *C. poecilopus*, males, females, juveniles, spawning, non-spawning) in thickness of epidermis and absolute volume of secretory cells, the method of hierarchical clustering was chosen using Euclidean distances and scale type standard deviation. The optimal clustering method (unweighted group average in the case of

epidermis relative thickness, and pair-group simple average in sacciform and goblet cells) was chosen according to the cophenetic correlation coefficient and delta (0.5) and delta (1) criteria using software NCSS 2007 (NCSS, LLC.) and STATISTICA 8.0 (Statsoft Inc., Czech Republic).

The significance of the differences between groups (*C. gobio* vs. *C. poecilopus*, males vs. females, spawning vs. non-spawning) was tested by means of a three-way ANOVA in software NCSS 2007. Data on relative epidermis thickness were square-root transformed prior to the analyses to conform to the assumptions of parametric tests. Data on relative volume of goblet and sacciform cells were transformed to proportions (by dividing them by 100) and subsequently square-root transformed prior to the analyses.

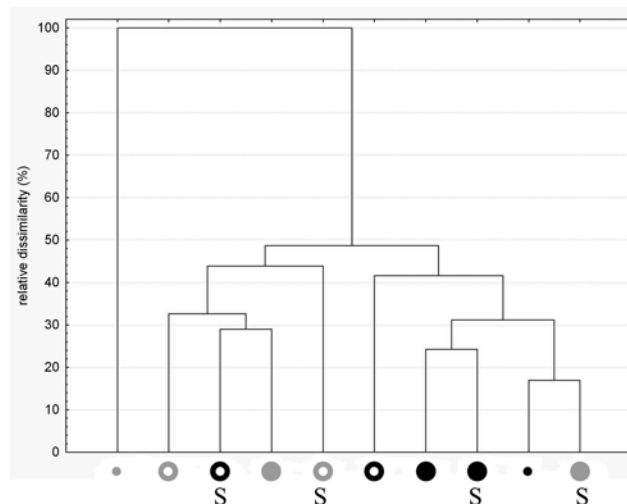


Fig. 1. Hierarchical clustering of *C. gobio* (grey) and *C. poecilopus* (black) groups according to relative thickness of the epidermis (males – full circle, females – empty circle, juveniles – spot, S – spawning period).

Results

Structure of the epidermis

The epidermis thickness in the head region (Table 3, Fig. 1) in the individuals studied ranged between 52 and 195 µm in *C. gobio*, and between 26 and 152 µm in *C. poecilopus*. The relative epidermis thickness of

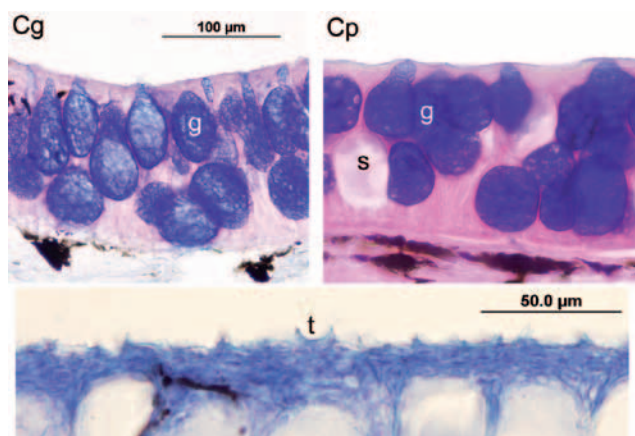


Fig. 2. Epidermis of *C. gobio* (Cg; male) and *C. poecilopus* (Cp; female) in the non-spawning period with goblet (g) and sacciform (s) cells, Alcian blue at pH 2.5. Sporadically found toothlets (t) on the surface of the epidermis (*C. gobio*, male), Mallory trichrome stain.

males, especially in *C. gobio* during spawning period, reached higher values compared to females ($p < 0.001$). In the dendrogram (Fig. 1), *C. gobio* males in spawning were in the same cluster with *C. poecilopus*, whereas the other adult *C. gobio* formed different cluster. Juveniles of *C. gobio* were situated outside the two main clusters differentiating the adults of *C. gobio* and *C. poecilopus*. The differences between species and spawning/non-spawning were not significant ($p = 0.427$ for species and 0.946 for spawning).

Similarly to other fish species, the epidermis of sculpins is divided into three basic layers – basal, middle and

superficial. The superficial layer of the epidermis consists of several layers of epidermal cells; in the surface layer, the cells may create small toothlets (Fig. 2). In the middle layer of the epidermis, in both species, there were two types of morphologically similar great secretory cells (these having a basal placed nucleus and a central secretive vacuole filling most of the cell, with a maximum height of 60-70 μm , and a width of up to 50 μm), which varied with regard to secretion compounds. Goblet cells were markedly alcianophilic and PAS positive, which corresponds with the glycoprotein character of the secretion. The second cell type was not positive to any staining method. The staining according to the Mallory and Papanicolaou method confirmed the predominance of protein. Such cells were considered to be sacciform cells.

With respect to each type of secretory cell, differences in quantity were observed (Table 4, Fig. 3), not only between sculpin species (the difference between species in goblet cells at $p = 0.0256$ and in sacciform cells at $p < 0.001$), but in the case of goblet cells also in relation to spawning activity ($p < 0.001$) and gender ($p < 0.001$). The most important finding was the higher volume of sacciform cells in *C. poecilopus* (*C. gobio* adults formed cluster far from the other groups in right dendrogram in Fig. 3), the next was the decrease in the number of both secretory cells in both species during the spawning period (in both dendrograms in Fig. 3 *C. poecilopus* individuals in spawning were situated in different clusters compared to those outside the spawning period).

Table 3. Absolute (μm) and relative ($\mu\text{m.cm}$ of SL^{-1}) thickness of the epidermis of males (M), females (F), and juveniles (J) of *C. gobio* and *C. poecilopus* outside and during the spawning period.

	Absolute thickness (μm)					Relative thickness ($\mu\text{m.cm}$ of SL^{-1})				
	spawning period		non-spawning period			spawning period		non-spawning period		
	M	F	M	F	J	M	F	M	F	J
<i>Cottus gobio</i>	123 \pm 48	88 \pm 41	102 \pm 19	84 \pm 10	79 \pm 8	14 \pm 2	10 \pm 3	12 \pm 2	11 \pm 2	18 \pm 3
<i>Cottus poecilopus</i>	82 \pm 11	82 \pm 11	120 \pm 17	100 \pm 17	70 \pm 5	12 \pm 2	11 \pm 2	13 \pm 2	12 \pm 2	14 \pm 1

Table 4. Relative (%) and absolute (abs) volume of goblet (GC) and sacciform cells (SC) in the epidermis of males (M), females (F), and juveniles (J) of *C. gobio*, and *C. poecilopus* during (sp) and outside (non-sp) the spawning period.

		%					abs				
		sp		non-sp			sp		non-sp		
		M	F	M	F	J	M	F	M	F	J
<i>Cottus gobio</i>	GC	25 \pm 9	15 \pm 11	49 \pm 12	40 \pm 9	46 \pm 4	34 \pm 23	17 \pm 18	51 \pm 19	33 \pm 8	36 \pm 6
	SC	0 \pm 1	0 \pm 1	1 \pm 1	1 \pm 1	3 \pm 2	0 \pm 1	0 \pm 1	1 \pm 1	1 \pm 1	3 \pm 2
<i>Cottus poecilopus</i>	GC	13 \pm 5	14 \pm 6	41 \pm 9	36 \pm 9	24 \pm 4	11 \pm 4	11 \pm 4	50 \pm 14	36 \pm 12	17 \pm 3
	SC	5 \pm 2	4 \pm 3	11 \pm 4	12 \pm 4	10 \pm 3	3 \pm 2	4 \pm 2	13 \pm 6	12 \pm 5	7 \pm 2

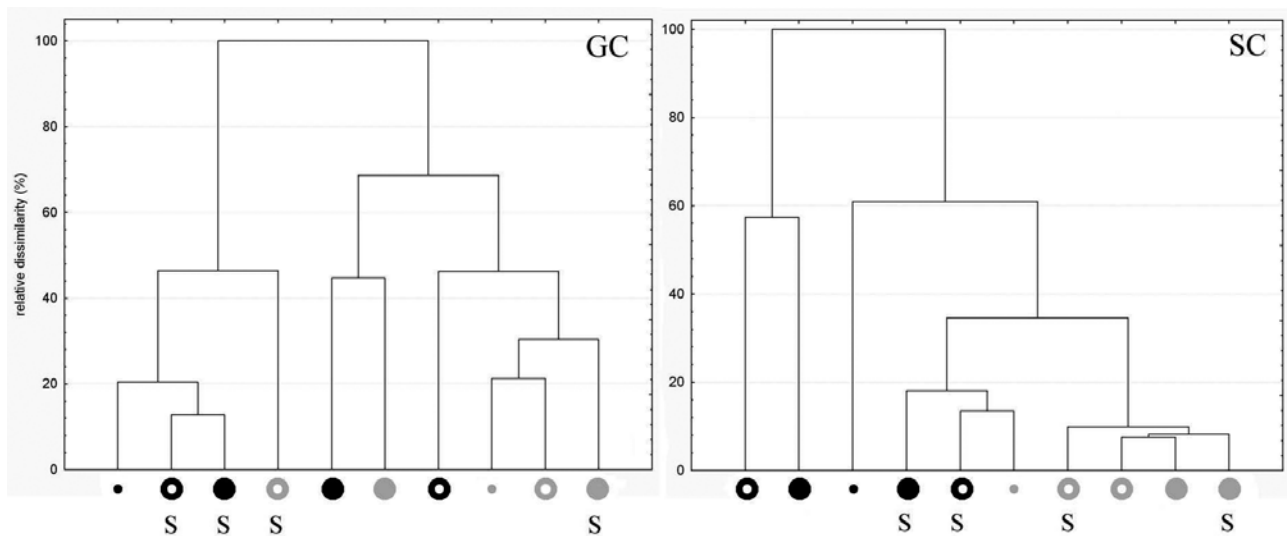


Fig. 3. Hierarchical clustering of *C. gobio* (grey) and *C. poecilopus* (black) groups according to absolute volume of goblet (GC) and sacciform cells (SC) (males – full circle, females – empty circle, juveniles – spot, S – spawning period).

Table 5. Erythrocyte and leukocyte counts (mean value and 95 % confidence interval according to (Horn 1983)) in *C. gobio* and *C. poecilopus* just after capture and after 72 hours exposure to increased water temperature and decreased oxygen saturation (LCL - lower 95 % confidence limit, UCL – upper 95 % confidence limit).

	variant	Erythrocyte (T/l)			Leukocyte (G/l)		
		LCL	mean	UCL	LCL	mean	UCL
<i>Cottus gobio</i>	unstressed	1.75	1.90	2.05	27.0	31.5	35.5
	stressed	2.49	2.56	2.62	52.5	61.5	70.0
<i>Cottus poecilopus</i>	unstressed	1.50	1.57	1.63	27.5	30.5	33.5
	stressed	2.39	2.66	2.90	48.0	51.5	54.5

Pigment cells, or melanophores, were commonly found in the middle part of the epidermis of both species.

Blood count

Obtained results (Table 5) showed a 21 % increase in the average erythrocyte count in *C. gobio* compared to *C. poecilopus* (the difference was confirmed by ANOVA at $p = 0.012$). Moreover, the stress exposure evoked a marked increase in both species (34 % in *C. gobio* and even 67 % in *C. poecilopus*; treatments were statistically different at $p < 0.001$).

In the leucocyte count, no differences were confirmed in non-stressed fish ($p = 0.294$), but the stress exposure caused a distinct increase, especially in *C. gobio* (103 % in average); the increase in *C. poecilopus* was 73 % (the difference between treatments was confirmed at $p < 0.001$).

Discussion

The relative thickness of epidermis in the dorsal part of the head is similar in both species (11-12 $\mu\text{m}/\text{cm}$ of

SL) and corresponds with data of Jakubowski (1963) in *C. gobio*. A similar thickness was measured in several other small fish species: *Barbatula barbatula* 98 μm (10 cm), *Gobio gobio* 86 μm (13 cm) (skin samples from head, non-spawning period, Knoz & Halačka 1991). Although the difference in epidermal thickness is supposed, a complex comparison is complicated namely due to the heterogeneity of methods used and the absence of additional information in particular authors.

Epidermal thickening was recorded in *C. gobio* males during the spawning period. This trend is considered to be a part of the “breeding dress” also observed in various fishes of various orders: Salmoniformes – e.g. *Salmo trutta* m. *fario* (Pickering 1977, Knoz et al. 1990); Cypriniformes – usually in connection with breeding tubercles, e.g. *Abramis brama*, *Chondrostoma nasus* (Knoz & Halačka 1991, Halačka 1995); Perciformes – e.g. in *Perccottus glenii*, there is obvious inflation on top of the head (Tsepkin 1977). Although *C. gobio* individuals exhibited slightly

thinner epidermis than *C. poecilopus* in this study, it is unlikely, that such a small difference can play important role in different ability of a gas exchange through the skin between both species.

As in other Perciformes, the epidermis of both *Cottus* sp. contained two types of unicellular secretory cells: goblet and sacciform. As mentioned above, one of the main potential functions of sacciform cells is to produce alarm substances analogous to the secretion of the club cells. Indirect proof of this function in *C. gobio* and *C. poecilopus* could be our finding of its reduction during the spawning period, which corresponds with club cell reduction (moreover accompanied by a decrease in the number of goblet cells) in the epidermis especially in males of some cyprinid fishes during such a period (Smith 1976, Halačka 1995).

The differences in total volume of sacciform cells between *Cottus gobio* and *Cottus poecilopus* can be related to different conditions in preferred habitats. With regard to the reproductive behaviour of sculpins (nest construction, biting of females by the male, egg-guarding and aerating (Morris 1954)), it is likely that epidermis damage could appear and that released alarm substances from sacciform cells could not only discourage the reproductive partner, but also inform predators about nest location. *C. poecilopus* prefers to inhabit upper spring parts of streams with lower diversity of fish communities. *C. gobio*, in contrast, prefers downstream parts of rivers with a variable habitats with other fishes and potential predators (e.g. *Salmo trutta*, *Salmo salar*, *Esox lucius*, *Anguilla anguilla*, *Leuciscus cephalus*, *Perca fluviatilis* (Andreasson 1969, 1972, Tomlinson & Perrow 2003)). Besides other mechanical counter-infection features, which are exhibited by diverse parts of particular types

of secretory cells, the lower proportion of sacciform cells in *C. gobio* can be related to a restriction on the release of signal substances, which is an advantage for fish which rely on hiding activities to escape predators. In the case of *C. poecilopus*, the predation pressure in preferred habitat is not too intensive.

On the other hand, in shallow streams at higher elevations, where UV radiation is more powerful, the higher rate of sacciform cells (together with the epidermal melanophores) can serve as a protective UVB barrier, mentioned by Chivers et al. (2007) and Blaustein & Mathis (2007).

Observed lower erythrocyte counts in *C. poecilopus* compared to *C. gobio* correspond with the higher oxygen demand of this species (Čihař 1969, Starmach 1971), but are in contradiction with data from Starmach (1970) on the same species in the River Raba (*C. gobio*, 1.54 T.l⁻¹) and the Mszanka stream (*C. poecilopus*, 1.93 T.l⁻¹). More information about various populations is needed. On the other hand, findings about the ability of both studied species to respond relatively rapidly to deteriorating environmental conditions by massively increasing their erythrocyte counts are in conformity with the mentioned author.

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