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Differences in haematology and respiratory system morphology at three neogobiid species

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Abstract: Gill characteristics and the size and number of erythrocytes were observed for three species of goby: *Neogobius melanostomus*, *N. fluviatilis* and *N. kessleri*. While erythrocyte size was similar in all species, *N. fluviatilis* had a statistically higher erythrocyte count. Significant differences were found in gill apparatus structure, with *N. melanostomus* having 25% and 50% greater gill contact area than *N. kessleri* and *N. fluviatilis*, respectively. Secretory goblet cells, which produce a protective mucous, were also present in highest numbers in *N. melanostomus* gill tissues. These physiological and morphological differences may be one reason for differences observed in the invasiveness of these three species.

Keywords: invasive species, goblet cells, oxygen transport, *Neogobius melanostomus, N. fluviatilis, N. kessleri*

1 Introduction

Fishes of the genus *Neogobius* are probably the most invasive group of fishes today, having taken over from *Carassius gibelio* or *Pseudorasbora parva* whose invasion wave progressed across the European continent in the second half of the last century [1,2].

In contrast to *C. gibelio*, however, neogobiids are stagnant and had little commercial value, meaning that natural expansion or anthropogenic introduction has been minimal; rather, the primary route of introduction has been passive, i.e. in ballast water in river and ocean-going ships. As such vessels are estimated to transport nearly 10 billion tonnes of ballast water world-wide annually [3,4], the potential for invasion is high. Neogobiids are currently estimated to represent at least a third of all fish species found in ballast water, with the dominant group consisting of just three species: *N. melanostomus*, *N. fluviatilis* and *N. kessleri* [5].

While all three species have a similar point of origin, largely overlapping areas of original occurrence and similar habitat requirements, invasion intensity and direction of progress have differed significantly. In terms of invasion potential, *N. melanostomus* appears to have been the most successful [6], which is also reflected in the increasing level of research on this species. While most studies have concentrated on biological aspects or on habitat preferences, however, relatively few have examined reasons for its invasion success [7,8].

A possible cause for the species' success may be through interspecific differences in physiology and/or morphology. Increased knowledge on the physiological and morphological properties of individual invasive organisms, including information on border values of survival, therefore, could significantly improve the success of such measures.

Identification of such differences in invasive species could provide information useful for restricting further expansion, e.g. in preventing transport in ship ballast water – the way the water is taken in (and released) and regulation its physicochemical parameters. Experience has shown that existing measures such as limiting the maximum size of particulate matter pumped on board with ballast water or relying on worsening ballast water quality over time is totally inadequate. For example, ballast tanks with a reported maximum particle suction size of around 1 cm have been found to contain fishes of up to 35 cm [9], while analysis of ballast water oxygen in ships at anchor in Hong Kong harbour revealed concentrations of around 4.8-10.7 mg/l, high enough to maintain many water organisms [10]. A range of more active intervention measures inside ballast tanks are now being considered, including acoustic techniques, electric and plasma pulse

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techniques, and thermal treatment [9]. Such measures, however, increase cargo shipping costs. As a result, there is growing pressure to determine minimum effective values in order to increase efficiency.

Respiratory system structure, which serves both for gaseous exchange and as a barrier against a range of environmental factors (such as salinity or infection), is probably the most important physiological and morphological attribute affecting species condition (metabolism), habitat selection, adaptation and survival in new environments and survival in lowered (especially hypoxic) oxygen conditions. The effectiveness of gaseous exchange is dependent also on circulatory system function, where it plays a key role, as well as surface area of erythrocytes. As such, the aim of this study is to identify and quantify interspecific differences in respiratory structure and erythrocyte characteristic between *N. melanostomus*, *N. fluviatilis* and *N. kessleri*.

2 Experimental Procedures

Two locations were chosen for obtaining experimental individuals:

i) The lower River Rhine (North Sea basin), near the town of Rees in Germany (N 51°45’36”, E 6°20’23”). Here, *N. melanostomus*, *N. kessleri* a *N. fluviatilis* occur sympatrically. Fishes were caught by electrofishing between 16 and 18.4.2012 (Table 1). Water temperature was 11°C and oxygen saturation was 94% (10.1 mg/l).

ii) The lower River Dyje (Black Sea basin), near the town of Břeclav in the Czech Republic (N 48°44’31”, E 16°53’32”). At this time, only *N. melanostomus* occur along the sample stretch (Table 1). Fish were caught using the same equipment and methods as mentioned above on 23/5/2012. Water temperature was 19°C and oxygen saturation 85% (7.2 mg/l).

Table 1. Number of fish sampled and mean standard length (in cm; standard deviations showed in brackets) for *N. melanostomus* (M_D = Dyje; M_R = Rhine), *N. kessleri* (K) and *N. fluviatilis* (F).

	MT	*MR*	*K*	*F*
Number sampled	15	21	15	15
Mean standard length	9.4	7.9	8.8	9.2
	(1.34)	(1.20)	(1.24)	(1.70)

2.1 Blood sampling and analysis

In order to assess the number of erythrocytes (red blood cells) in the blood, samples were taken from the caudal vein [11,12] of 10 individuals of each species, dilute 1:199 with the Natt and Herrick’s solution and count in Bürker chamber.

At the same time, blood smears were also taken (air-dried and stained in Hematoxylin Solution, Harris Modified (Sigma-Aldrich)), allowing assessment of maximum (E_{max}) and minimum (E_{min}) erythrocyte size (30 erythrocytes from each individual), images were captured by CCD camera Olympus DP70 with microscope Olympus BX 50 and processing by Olympus MicroImage 4.0 software. Surface area was calculated using the formula

$$E_{area} = \pi \times E_{max} \times E_{min} / 4.$$

2.2 Gill morphology

Gill morphology was assessed on seven individuals from each species group. Three measurements were taken to assess total gill contact area: 1) sum of the lengths of all individual primary lamella filaments on the lateral series of the first right gill arch (Σpl), 2) the distance between secondary lamellae on the primary lamella (Dsl), and 3) the maximum length of the secondary lamellae from the middle of the first right gill arch (Lsl). Gill contact area was calculated as Σpl / Dsl . Lsl and the result divided by fish standard length (SL, in cm; Table 1) to provide gill area per 1 cm SL, allowing comparison between species groups [13]. All measurements were taken from fixed specimens following removal of the gill arch.

Primary lamellae from the middle of the branchial arch were also used for histological incisions. In order to detect mucous goblet cells and assess the composition of secretory cell content, individual lamellae were stained using mucinophilic Alcian blue at pH 1.0 for sulphated acid glycoproteins and at pH 2.5 for acid glycoproteins [14], and with Periodic acid-Schiff for neutral and acid sialylated glycoproteins [15].

2.3 Statistical analysis

Differences in erythrocyte count, erythrocyte area and gill area between the Dyje and Rhine populations of *N. melanostomus* were revealed using t-tests, and those among Rhine populations of *N. melanostomus*, *N. kessleri* and *N. fluviatilis* using analysis of variance (ANOVA, with Scheffé *post-hoc* tests). The data indicated a relatively normal and homogenous variance with one exception, the comparison of erythrocyte area between Dyje and Rhine *N. melanostomus* populations which showed normal distribution but unequal variances. These fish were tested, therefore, using the t-test modified for unequal variance (Welch approximation to the degrees of freedom).

3 Results

The erythrocyte count observed in the Rhine *N. fluviatilis* population was significantly higher than that for both *N. kessleri* and *N. melanostomus* (ANOVA, $F_{2, 45}$ =22.98, $p < 0.001$; Scheffé test, both $p < 0.0001$; Table 2; Figure 1). No significant differences were observed between *N. melanostomus* and *N. kessleri* (Scheffé test, $p > 0.05$). Similarly, no difference was observed between Dyje and Rhine populations of *N. melanostomus* (t-test, $t_{1, 34} = 1.77$, $p > 0.05$).

On the other hand, erythrocyte area for the Rhine *N. kessleri* population was significantly higher than that for both *N. melanostomus* (ANOVA, $F_{2, 27} = 8.61$, $p < 0.01$; Scheffé test, $p < 0.01$) and *N. fluviatilis* (Scheffé test, $p < 0.001$; Table 2; Figure 1). No significant differences were observed between *N. fluviatilis* and *N. kessleri* (Scheffé test, $p > 0.05$), however, nor between Dyje and Rhine populations of *N. melanostomus* (t-test modified for unequal variances, $t_{1, 4.62} = 1.01$, $p > 0.05$).

Gill area, expressed as length of secondary lamellae per 1 cm SL, showed distinct interspecific differences (ANOVA, $F_{2, 18} = 4.93$, $p < 0.05$; Table 3; Figure 2), with *N. melanostomus* gill area up to 50% greater than that of

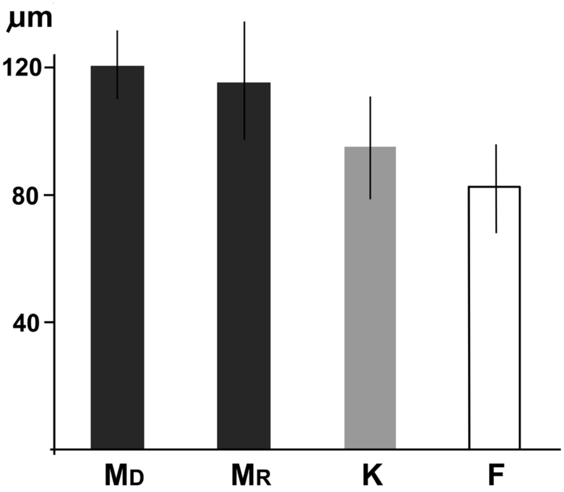

Figure 1. Erythrocyte count and erythocyte area for *N. melanostomus* (M_D = Dyje; M_R = Rhine), *N. kessleri* (K) and *N. fluviatilis* (F); error bars show 95% confidence intervals.

Table 2. Comparison of mean values (standard deviations showed in brackets) for erythrocyte profile for *N. melanostomus* (*MD* – Dyje; *MR* - Rhine), *N. kessleri* (*K*) and *N. fluviatilis* (*F*). Erythrocyte count statistics are based on samples of 15, 21, 12 and 15 fish (for *MD*, *MR*, *K* and *F*, respectively). Erythrocyte size statistics are based on samples of 5, 10, 10 and 10 fish.

		MD	*MR*	*K*	*F*
Erythrocyte count	T/l	1.66	1.56	1.61	1.92
		(0.13)	(0.18)	(0.11)	(0.15)
Erythrocyte major axis	µm	12.2	12.3	13.0	12.9
		(0.60)	(0.32)	(0.42)	(0.57)
Erythrocyte minor axis	µm	8.6	8.9	9.0	8.3
		(0.53)	(0.31)	(0.37)	(0.20)
Erythrocyte area	μm^2	81.2	86.3	92.3	84.5
		(8.02)	(3.31)	(5.12)	(4.04)

Table 3. Comparison of mean values (standard deviations showed in brackets) for gill profile for *N. melanostomus* (*MD* – Dyje; *MR* - Rhine), *N. kessleri* (*K*) and *N. fluviatilis* (*F*). (based on samples of 7 individuals of each species); Σ pl - sum of the lengths of all individual primary lamella filaments on the lateral series of the first right gill arch, Dsl - distance between secondary lamellae on the primary lamella, Lsl - maximum length of the secondary lamellae from the middle of the first right gill arch, GCA - gill contact area, GCA/SL - gill contact area calculated per 1 cm SL.

		MD	*MR*	*K*	*F*
Σ pl	mm	99	101	91	68
		(10.4)	(13.2)	(10.0)	(7.2)
Dsl	mm	0.071	0.069	0.062	0.050
		(0.0037)	(0.0041)	(0.0045)	(0.0051)
Lsl	mm	0.73	0.66	0.55	0.51
		(0.068)	(0.101)	(0.067)	(0.059)
GCA	mm	1 011	955	818	705
		(103)	(179)	(170)	(134)
GCA/SL	mm/cm	120	115	95	82
		(12.7)	(21.2)	(17.4)	(14.9)

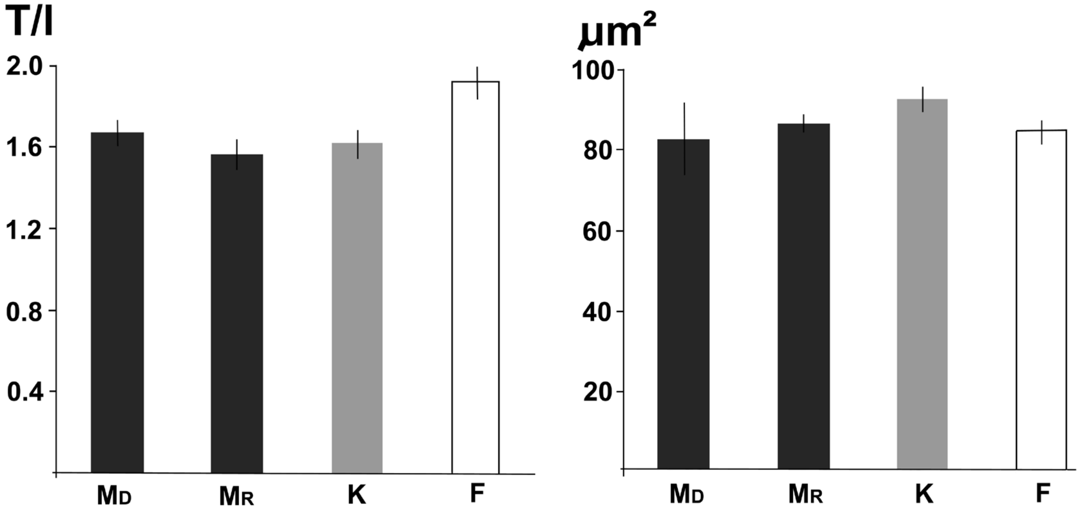

Figure 2. Mean relative length of secondary lamellae on the first gill arch of *N. melanostomus* (M_D - Dyje, M_R - Rhine), *N. kessleri* (K) and *N. fluviatilis* (F); error bars show 95% confidence intervals.

N. fluviatilis (Scheffé test, $p < 0.01$). No other significant differences were observed in Rhine populations (Scheffé tests, $p > 0.05$). In addition, no difference was observed between Dyje and Rhine populations of *N. melanostomus* (t-test, $t_{1, 12} = 0.52$, $p > 0.05$).

Individual species differences were also observed in representation of secretory goblet cells in gill tissue, with goblet cells common in *N. melanostomus* (no significant differences were recorded between the two *N. melanostomus* populations studied) and moderate or rare in the remaining two species (Figure 3). Histochemical staining indicated a stable mixture of neutral, acid sialylated and acid (including sialylated and sulphated) glycoproteins in *N. melanostomus*; while results were more variable in *N. kessleri* and *N. fluviatilis*, with either neutral or acidic glycoproteins tending to dominate.

4 Discussion

Maintaining an adequate oxygen supply is one of the most important requirements of living organisms. In contrast to land-based organisms, aquatic organisms are exposed to much greater fluctuations in oxygen supply

Figure 3. Goblet cells on the gills of *N. melanostomus* (M), *N. kessleri* (K) and *N. fluviatilis* (F). Samples stained with Alcian blue at pH 2.5.

as the inherent properties of water can result in marked spatial and temporal differences in oxygen concentration (hypoxia). To address this, two main adaptive mechanisms have evolved, i.e. interspecific differences in erythrocyte count and gill morphology [16].

The number of erythrocytes in the bloodstream is closely related to the level of oxygen transported and it is possible find some relationship between their number and resistance of fish species to hypoxia. Fishes living in the upper water column or in fast-flowing waters, where oxygen levels tend to be higher, have lower erythrocyte concentrations (e.g. *Salmo trutta* - 1.08 T/l [17]; *Thymallus thymallus* - 1.29 T/l [18]; *Salvelinus fontinalis* - 1.13 T/l (Halačka, unpublished data)) than those from deeper or slow-flowing waters with lower oxygen levels (e.g. *Barbus barbus* - 1.90 [19]; *Chondrostoma nasus* - 1.85 T/l [20]; *Carassius gibelio* - 1.69 T/l [12]. Likewise, Halačka [21] recently found that two Cottidae species sampled from the same site had differing erythrocyte counts, with the more tolerant *Cottus gobio* having a higher count (1.93 T/l) than the more specialised *C. poecilopus* (1.57 T/l). From this perspective, *N. fluviatilis* appears to be better adapted to lower oxygen concentrations than *N. kessleri* and *N. melanostomus*.

The number of erythrocytes is very important, but so is their size. Smaller erythrocytes have larger surface area to volume ratios and shorter diffusion distances allowing more rapid oxygen transfer [22]. That confers better potential efficiency to gaseous exchange *N. fluviatilis*, which has a total erythrocyte area (erythrocyte count x area) larger (162 m^2/l) than at *N. kessleri* (149 m^2/l) or *N. melanostomus* (136 m^2/l (Dyje); 134 m^2/l (Rhine)).

Haematology and resistance to hypoxia in *N. melanostomus* has also been examined [23-25], using fish sampled from their original area of occurrence, i.e. Sevastopol Bay in Ukraine. Similar to our results, these authors reported erythrocytes size as 12.6 × 8.7 μm (major × minor axis). In addition, they also reported erythrocyte counts from individuals reared under both normal (1.04 T/l) and hypoxic conditions (1.16 T/l), both of these values being considerably lower than those obtained from this study.

Erythrocyte numbers do not necessarily remain constant; many animals are able to increase their erythrocyte count dramatically as a reaction to stress, e.g. low oxygen levels. As an example, two members of the genus *Cottus* (*C. gobio* and *C. poecilopus*) were able to significantly increase their erythrocyte numbers (in some cases up to twice its original value) in just a few tens of hours during a 20% decrease in oxygen saturation [26,27]. In *N. melanostomus*, however, a reduction in oxygen saturation from 100% to 20% (< 2 mg/l) only resulted in an increase in erythrocyte number of around 12% [23-25]. This relatively low reaction may indicate the ability of *N. melanostomus* to secure adequate levels of oxygen, even at low oxygen concentrations. On the other hand, the high erythrocyte values found in the invasive individuals in this study (compared with those from its original area of occurrence) certainly indicates an ability to increase erythrocyte count. This raises the question as to why it was so high in the invasive fish but not the non-invasive individuals. If the lower counts in the non-invasive individuals can be considered universal, then perhaps the higher counts found in the invasive individuals from the Rhine and Dyje represent an adaptation to new climatic and hydrological conditions in the newly settled territory.

In addition to erythrocyte count, a fish's ability to occupy a specific environment and/or tolerate hypoxia will depend to a large degree on gill system morphology [28,29]. Gill apparatus area and the number of filaments are related to, among other things, the species' preferred habitat, with fishes living in waters rich in oxygen tending to have lower gill area [30]. From this perspective, the differences found in the length of secondary gill lamellae in this study are clearly important, the ca. 50% increase in *N. melanostomus* gill contact area representing a significant advantage for survival in ballast tanks and better acclimatisation in new environments. This, combined with the species' tolerance to hypoxic conditions up to 0.3 mg/l [31] and its high temperature tolerance (-1.0 - +32°C; [32,33]), probably explains its primary role in this invasive group. Such high tolerance levels could render planned preventive measures by ship owners ineffective, e.g. through thermal treatment of ballast water [9].

In addition to gaseous exchange, the gill apparatus also plays a primary role in ion and acid-base regulation, nitrogen excretion and in resistance to parasites and infections [34,35]. In this case, gill morphology also plays an important role, and particularly the representation of functional cells such as chloride cells and the multifunctional goblet (or mucous) secretory cells. Unfortunately, these are frequently difficult to detect, usually requiring electron microscopy or immunochemistry techniques [36,37].

Mucous cells are commonly found in the epidermal, gill and intestinal epithelium [29,38], their frequency and secretory composition differing both between species and within species, or even within individuals, depending upon environment or individual body condition [38-40].

During the ballasting process, fish taken on board may be subject to sudden changes in salinity, and again during the offloading of ballast water [41]. Such changes in salinity are detrimental to fish as it can result in a loss of internal water or ions [36,37].

Mucous appears to play a protective role this process [36], hence the occurrence of high numbers of mucous-producing goblet cells, as observed on *N. melanostomus* gills in this study, may provide the species with a distinct survival and invasive advantage.

In addition, the gill's mucous layer has been shown to affect resistance to amoebic gill disease [42] and may also help combat bacterial and fungal infections [34,43]. Studies appear to indicate that a combination of both neutral and acidic glycoproteins is most advantageous, as found in *N. melanostomus* in this study, as neutral mucosubstances have been shown to have a protective role [34]. Sulphated glycoproteins, on the other hand, appear to be more effective in freshwater osmoregulation [43].

Finally, the mucous cover protects the fish against drying out, and thus inhibits reduction of respiration. This is of importance for fish living in habitats with fluctuating water levels, such as along coastal cliffs, regulated rip-rap banks or in ship ballast tanks, where individuals may be exposed to the air [44].

To conclude, *N. melanostomus* shows specific physiological and morphological differences that provide the species with a high degree of tolerance to adverse environmental conditions, which may help explain its significant invasive success compared to other Gobiids.

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