Sexual trait may simultaneously indicate sperm production and nutritional fitness in uniparental nest guarding fishes: a case study on Amur sleeper

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Short title: Secondary sexual trait and male fitness

Abstract

The reproductive success of uniparental males depends much on their nutritional stage (i.e. body condition). Therefore, secondary sexual traits should convey information not only on fecundity but also on nutritional stage. To test this hypothesis, we evaluate relationships between head crest height (HCH), as a secondary sexual trait, relative testes mass (an indicator of sperm production), body condition (an indicator of nutritional tatus) and body mass of males of uniparental Amur sleeper *Perccottus glenii* in two contrasting habitats. Furthermore, we examined whether head crest is only a pure investment to advertise male fitness – either to females, rival males or other fish approaching the nest – or it comprises some regainable energy as well. We found that HCH is closely related to relative testes mass, and body condition especially at the beginning of the reproductive period. The head crest consisted of a swollen connective tissue in the supraorbital region of the head, and an oedematous epaxial muscle in the nuchal region. Further, the head crest comprised adipose tissue as well, although not that much as assumed. Therefore, we argue that HCH represents a reliable signal of multiple aspects of male quality; it indicates readiness to spawn and could potentially effectively facilitate female choice towards large males with high fecundity and great nutritional stage, which is essential to good guarding performance. Moreover, the head crest itself also could contribute to nest guarding performance by virtually enlarging body size and by representing a minor fat reserve.

Keywords

head crest; nuchal hump, Fulton’s condition factor; gonadosomatic index; body size; rotan

Introduction

In animals, mate choice is a common phenomenon, since females may benefit (i.e. high fertilisation rate, good genes for offspring, food and parental care) by choosing high quality males (Andersson, 1994; Pizzolon *et al.*, 2012). Therefore, males in several species develop conspicuous secondary sexual traits (SSTs) to advertise their quality and to acquire mates (Simmons, Lüpold & Fitzpatrik, 2017; and references therein).

SSTs can signal for different aspects of reproductive performance. In non-resource-based mating systems, where males provide just sperm, SSTs are expected to reflect functional fertility and genetic condition (Andersson, 1994; Sheldon, 1994; Pitcher & Evans, 2001). In species, where the male cares for the offspring, however, SSTs should also convey information on parental ability and general fitness (Birkhead, Fletcher & Pellatt, 1998; Pizzolon *et al.*, 2012). During the parental phase – especially when males are guarding the nest – they frequently face food deprivation and substantial reduction in body condition; thus, their nutritional fitness (i.e. resource acquisition and assimilation efficiency) may strongly relate to parental ability (Pike *et al.*, 2007; Locatello, Pizzolon & Rasotto, 2012). The association between SST and nutritional stage is especially important in uniparental species (Pizzolon *et al.*, 2012), because the nutritional fitness of nest guarding males can be the cornerstone of their reproductive success. Hence, we can hypothesize that SSTs can indicate the current nutritional stage as well as fecundity in species exhibiting uniparental care.

During the reproductive season, Amur sleeper *Perccottus glenii* Dybowski 1877 (Pisces, Gobiiformes, Odontobutidae) males display a marked SST, the head crest, an enlarged tissue area at the top of the head (Fig. 1). Amur sleeper males invest much energy to guard their nest aggressively and to take care of the fertilized eggs (fanning) until hatching (Bogutskaya & Naseka, 2002). The head crest is considered to provide a more robust appearance for males, which is beneficial in nest guarding (Baerends & Baerends-Van Roon, 1950). However, it is not clear what information the head crest could provide for females or rival males, and whether it could contribute to nest guarding performance directly as an energy reserve, as well. Accordingly, we aimed to examine relationships between the head crest height (HCH) and nutritional status, sperm production and body size of this freshwater fish. Due to its energetic cost, it is likely that development of the head crest is influenced by both the fitness (genetic and nutritional), and the environmental conditions (Cotton, Fowler & Pomiankowski, 2004). Therefore, we investigated two contrasting habitats, a eutrophic oxbow lake, and a lowland canal. Because Amur sleeper prefers densely-vegetated standing waters (Kottelat & Freyhof, 2007), we supposed that the latter habitat could provide less favourable conditions for maintaining a high nutritional status. Accordingly, we predicted that in Amur sleeper males: i) HCH positively associates with body condition (an indicator of nutritional stage); ii) HCH also positively associates with relative testes mass (an indicator of sperm production); iii) HCH changes in time with reproduction activity; and iv) HCH differs between the oxbow lake and the canal habitats. Further, we assumed that development of the head crest may concur with energy accumulation for active nest guarding, thus we supposed that: v) it should not be a pure advertiser of male fitness, but also an energy reserve. Therefore, we examined the tissue composition of the head crest, and focused especially on the presence of lipids and adipose tissue.

Material and methods

The Amur sleeper is a small sized, predatory fish species native to Far-East Asia. It is considered invasive in Europe where it has recently established populations in many areas (Reshetnikov, 2013; Kati *et al.*, 2015; Nehring & Steinhof, 2015; Covaciu-Marcov, Ferenţi & Sas-Kovács, 2017). The species spawns in late spring, above 15°C water temperature (Kottelat & Freyhof, 2007; Grabowska *et al.*, 2011).

We examined two habitats: a eutrophic oxbow lake (N48.095889, E21.462722) with a 0.78 km^2 surface area and 0.7 m mean depth; and a lowland canal (N48.144056, E21.629750) which is 91 km long and 6–7 m wide, its depth ranges between 1–2 m and velocity between 15–55 cm s^{-1}.

Fish were collected by electrofishing monthly between May and July in 2011, on three sampling occasions in both habitats. The collection was approved by the Agricultural, Public Administration Office of Hungary (permission number: 04.3/440-3/2011.). We restricted our study to the May to July period, because later in the year HCH values of males decrease to a value similar to that of females. Captured fish were euthanized immediately with an overdose of clove oil and preserved in 5% formaldehyde. At each sampling occasion we collected at least 50 specimens, resulting in 29, 24 and 14 males from the oxbow lake and 25, 29 and 25 males from the canal in May, June and July, respectively. In the laboratory we measured head crest, total (TL) and standard length (SL) to the nearest 0.1 mm, and body mass (BM) to the nearest 0.01 g, using digital caliper and balance. For measurement, we defined head crest as the orbital (at the centre of the eye) height of the head. Actually, we measured the highest vertical extension of the frontal part of the head crest, the supraorbital hump (more detail is provided in the results), which is also the most variable attribute of the head crest both seasonally and between individuals. Then, fish were dissected to identify their sex and to measure the mass of testes in males to the nearest 0.0001 g. For analyses, we standardised the head crest height for TL and hereafter referred as HCH (HCH = head crest (mm) × TL $(mm)^{-1}$). We expressed relative testes mass as gonadosomatic index (GSI = 100 × testes mass (g) × BM $(g)^{-1}$) and we used Fulton's condition factor to characterise body condition, hereafter referred as K (K = 100 × BM (g) × TL $(mm)^{-3}$; Nash, Valencia & Geffen, 2006).

For general histology and detection of lipids and adipose tissue in the head crest, supplementary sampling was performed in an oxbow lake in 2020 (permission number:

HaGF/113/2020). Fish (3 females, 3 males with conspicuous head crest) were euthanized then, their heads were cut and fixed three days at 4°C in 0.1 M phosphate buffer (PB, pH = 7.4) containing 4% paraformaldehyde. Pieces of fixed head crest were cut into 16 μm thick serial sections with a cryostat, dried onto superfrost ultra plus (Thermo Scientific) glass slides, dehydrated in graded ethanol and xylene. Series of dehydrated sections were partly treated for alcian blue – Periodic Acid Schiff (PAS) staining (Sigma, periodic acid solution – 101646/1, Schiff's reagent – 101646/2, and alcian blue solution – 101647.05), partly used for haematoxylin-eosin (H-E; Sigma, 105174.05) staining. Sections of head crest were analysed by a Zeiss Axioplan compound microscope.

The relationship between HCH (response variable) and potential explanatory factors, season (May, June and July as dummy variables), habitat (oxbow and canal as dummy variables), BM, GSI and K were evaluated by multiple linear regression analysis using forward stepwise variable selection procedure (at $P < 0.05$) followed by variance partitioning (Cushman & McGarigal, 2002). Correlation between the explanatory variables was low to moderate (Pearson r ranged between 0.009 and 0.578). In order to test the interaction between the continuous explanatory factors (BM, GSI and K) we also included their second degree interaction terms to our preliminary model. However, since none of the interaction terms passed the variable selection test, they were not included in the final model. Assumptions of linearity and normality were checked visually by looking at the scatter plot of model residuals against each explanatory variable and the histogram of model residuals, respectively. Since both assumptions were met, there was no need to transform any of the numeric variables (BM, GSI, K). Since season proved to have a substantial influence on HCH, separate multiple linear regression analyses were executed by sampling occasions as well. Multiple linear analysis was performed by using Statistica 6.0 (StatSoft©) software.

Results

Full multiple regression model identified strong month (represented by May as dummy variable), and K and GSI related effects in the development of head crest in male Amur sleeper (Table 1). The effect of K was highly independent of the effect of GSI and season, whereas effects of the latter two factors overlapped greatly (Fig. 2). While fish size (expressed as BM) and habitat proved to have no influence on HCH at this scale. The full model explained 60.6% of the total variance in HCH.

HCH was more predictable in May than in June and July, with 65.0%, 35.1% and 36.0% total explained variance in the concerning models (Table 1). All monthly models included K, and co-predictors were the BM in May, and the GSI in June and July. Effect of BM and K overlapped considerably (50.3% of the total variance in HCH was explained as shared effect) in the May model, whereas in June and July model factor effects were largely independent.

The histology revealed that head crest consists of two markedly different parts (Fig. 3, 4). Based on their positions, we use the term suborbital hump for smaller swelling in the suborbital region, and nuchal hump for the larger, caudal part of the head crest (see Fig. 3 A2). The thickness of hypodermis in head crest region was much more swollen in males than in females due to heavily vascularisation, and many lipids (Fig 3). In the supraorbital region (Fig. 4 A), the hypodermis swollen due to physiological oedema. The suborbital hump is composed of a loose collagen type connective tissue (Fig. 4 I/a). This extremely swollen hypodermis layer contains many lipid vacuoles (Fig. 4 I/b), Wharton’s jelly-like tissue with different mesenchymal cell types, and fibroblasts (Fig. 4 I/c, -I/d). The nuchal hump is composed of watery epaxial muscle and focal massive adipose tissue (Fig. 3A, 4 II/a, II/b).

Discussion

The SSTs considered as visual cues of multiply aspects of male quality (Andersson, 1994; Sheldon, 1994; Birkhead, Fletcher & Pellatt, 1998; von Schantz *et al.*, 1999; Uetz, Papke & Kilinc, 2002). In species with parental care, the nutritional stage of guarding mate is one of the most important measures of quality (Sargent, 1992; Whoriskey & FitzGerald, 1994; Pizzolon *et al.*, 2012). By reporting positive association between head crest height (HCH), a marked SST in nest guarding male Amur sleeper, and body condition and relative testes mass, our study underlines the role of SST in advertising of nutritional stage and potential fertility. Beyond the communicatory function, we histologically evaluated the potential but minor contribution of the head crest to energy supply during nest guarding.

The nutritional stage is an important cue of mate quality, especially in species with parental care, because the poor nutritional stage could directly reduce mate performance and reproductive success (e.g. Sargent, 1992; Whoriskey & FitzGerald, 1994; Erikstad *et al.*, 1997; Olsson, Kvarnemo & Svensson, 2009). Therefore, we hypothesised (i) that the head crest should convey information on nutritional stage in uniparental Amur sleeper males. The positive association between the HCH and the body condition through the entire reproduction season confirms the hypothesised role of SST in advertising the nutritional stage, as a crucial measure of male quality in species with parental care. This positive association suggests that similarly to body condition, HCH is a nutrient dependent SST (Birkhead, Fletcher & Pellatt, 1998) and interindividual differences in resource acquisition or assimilation efficiency influence these two individual attributes in the same direction (Reznick, Nunney & Tessier, 2000). However, it should be noted that the relationship between the nutritional stage (expressed as foraging history or size of energy reserves) and parental performance is not always evident (Lindström & Sargent, 1997; Lindström, 1998; Lehtonen & Lindström, 2007). Hence, Amur sleeper males with more conspicuous HCH are not necessarily better father and its role in mate choice is doubtful.

The relationship between SSTs and male fertility is generally positive in fish (e.g. Pike *et al.*, 2010; Pizzolon *et al.*, 2012) and in the animal kingdom (Sheldon, 1994; Simmons, Lüpold & Fitzpatrik, 2017; but see Mautz, Møller & Jennions, 2013). In accordance with our related hypothesis (ii), HCH positively correlated with the GSI in Amur sleeper males. Similarly, as assumed (iii), the HCH was a function of time even within the reproductive season. Moreover, the amount of information (i.e. percent of variance explained) represented by the HCH was highest at the beginning of the reproductive period, in May, and decreased substantially later on. In other fish species, the SSTs can promote sex recognition (Barlow & Siri, 1997) and advertise the readiness to spawn (Amundsen & Forsgren, 2001; Clotfelter, Curren & Murphy, 2006). Based on its temporal and sex-specific aspects, the head crest could play similar roles in the Amur sleeper. The SSTs, in general, are under androgen control (Birkhead, Fletcher & Pellatt, 1998; Nelson 2005) which can ensure the link between the head crest and readiness to spawn. Sex hormones, furthermore, promote the sperm production (Borg, 1994; Måsvær, Liljedal & Folstad, 2004), supporting a positive association between the relative testes size (GSI) and HCH in Amur sleeper. Although, the relative testis mass is a reliable proxy of sperm production, it reflects only the potential fecundity (Simmons, Lüpold & Fitzpatrik, 2017), further studies should focus on the link between head crest and sperm quality.

Since nutritional stage dependent SSTs can be a function of environmental factors (Cotton, Fowler & Pomiankowski, 2004; Wong, Candolin & Lindström, 2007), we also hypothesised (iv) a habitat-related aspect of HCH. Although the two studied habitats differed considerably, analyses did not reveal an environmental influence. This finding may indicate the robustness of HCH as a SST and the interindividual variance in nutritional fitness ensure the positive relationship between SST and current nutritional status even within wide environmental scales.

Body size *per se* is an important cue of mate quality (Reynolds & Gross, 1992; Pitcher & Evans, 2001), especially in nest-guarding species (Noonan, 1983; Keenleyside, Rangeley & Kuppers, 1985; Wiegmann & Baylis, 1995; but see Forsgren, 1997). Based on the positive association between HCH and body size in May, the peak period of reproduction, this SST could promote the recognition of larger and more effective nest-defender males in Amur sleeper. Since HCH is a standardised measure of the head crest, this finding indicates that larger males have more pronounced SST not only in absolute but also in relative sense. A life-history study of Amur sleeper suggested that larger males spawn first (Grabowska *et al.*, 2011). This phenomenon supports the vital role of body size in mate acquisition and spawning territory occupation. Since the head crest increases frontal size appearance, larger males with larger head crest may benefit from this SST twice: by the advertisement of their superior size and by an enlarged virtual size. Therefore, head crest could play an important role in nest-defence (Baerends & Baerends-Van Roon, 1950) and male-to-male contests (Fabre *et al.*, 2014).

The soft and nonpitting characters of head crest in fish suggest its fat deposit origin (Patzner & Seiwald, 1987; Bleick, 1975). However, exact data on fat content of head crest are still insufficient or based on indirect assumptions such as the thickness of hypodermis (Takahashi, 2018; Infante *et al.*, 2018). Our analyses showed that in Amur sleeper male, the head crest histologically divides into two strictly different parts; the frontal part (supraorbital hump) develops mainly from lose connective tissues, while the caudal part (nuchal hump) composed mainly of muscle tissues. Contrary to our hypothesis (v), histological analysis revealed that swelling of both parts is mainly due to oedema and not to an accelerated fat deposition. This finding is in accordance with the results on Midas cichlid – which exhibits conspicuous crest prior to spawning (Barlow & Siri, 1997) – that also identified oedema as the main mechanism causing the rapid development of the crest (Bleick, 1975). Therefore, it is evident that the primary role of the head crest is not to fully compensate the effect of starvation during the nest guarding, but probably to advertise specific aspects of male fitness. Nevertheless, since lipids and some adipose tissues present in both parts of the head crest, this SST could also represent a minor supplementary energy reserve, and accordingly, may contribute little to longer and more efficient nest guarding as well.

To conclude, our findings highlight that the head crest of Amur sleeper males conveys information about the nutritional stage (potential guarding performance) and the relative testes mass (potential fertility), but may have only a limited energy reserve function for the period of the nest guarding. Although, the HCH could effectively facilitate female choice towards high quality males, the exact role of head crest size in mate choice is still a subject of debate. Further laboratory studies are needed, therefore, to reveal whether males with more characteristic head crest attract females that are more fecund, and whether females that mate with those males realize higher proportion of hatched larvae.

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Conflict of interest. The authors declare that they have no conflict of interest.

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Table 1. Sample size (n), body size (total length; TL, mm), statistics and model parameters (b) of multiple linear regression analysis on the influence of season (May to July), habitat (oxbow vs. canal) and individual traits (body mass, BM; gonadosomatic index, GSI; Fulton's condition factor, K) on head crest height in male Amur sleeper *Perccottus glenii*. Only significant (forward stepwise selection at $P < 0.05$) explanatory factors were retained in each regression model (full model including season, and separate models by sampling months).

			Model statistics				Regression coefficient				Explained variance (%)	
Model	n	TL: mean (range)	d.f.	*F*	*P*	$R^2_{adj.}$	b	SE	*t*	*P*	total	as pure effect
Full model	146	63.1 (33.5 – 127.2)	3; 142	75.3	<0.001	0.606					60.6	
Intercept							0.052	0.007	7.7	<0.001		
May							0.015	0.002	7.8	<0.001	25.5	16.4
GSI							0.009	0.002	4.5	<0.001	16.8	5.3
K							0.043	0.004	10.6	<0.001	24.5	30.6
May model	54	55.4 (33.5 – 70.0)	2; 51	50.1	<0.001	0.650					65.0	
Intercept							0.063	0.013	4.8	<0.001		
BM							0.005	0.002	2.8	0.007	54.9	4.6
K							0.043	0.011	4.0	<0.001	60.4	10.1
June model	53	64.3 (43.7 – 107.0)	2; 50	15.0	<0.001	0.351					35.1	
Intercept							0.087	0.008	11.3	<0.001		
GSI							0.005	0.002	2.4	0.018	1.7	6.4
K							0.023	0.004	5.2	<0.001	28.7	33.4
July model	39	72.2 (41.5 – 127.2)	2; 36	11.7	<0.001	0.360					36.0	
Intercept							0.081	0.011	7.4	<0.001		
GSI							0.011	0.003	4.0	<0.001	20.0	25.6
K							0.022	0.007	3.2	0.003	10.4	16.0

Figure 1. Amur sleeper *Perccottus glenii* males display a marked secondary sexual trait, crest at the top of the head during the reproduction season. (Photo: Ákos Harka)

Figure 2. Results of variation partitioning on the influence of habitat, season and individual traits (gonadosomatic index, body size and condition) on head crest height, a temporal secondary sexual trait of Amur sleeper *Perccottus glenii* males.

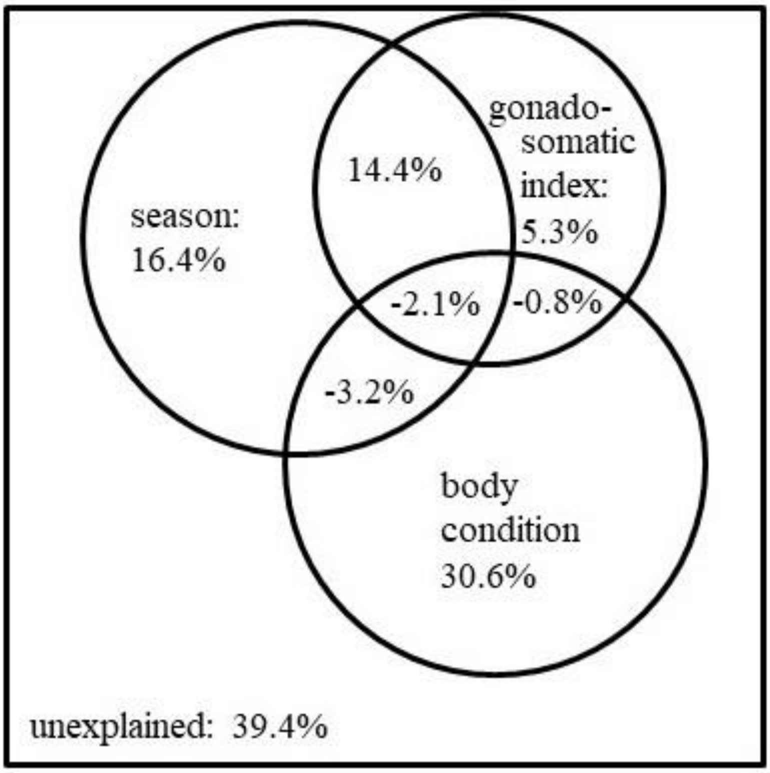


Figure 3. Vertical sections of the head crest of Amur sleeper *Perccottus glenii*. An overview native picture about the head crest of female (A1) and male (A2) during the reproduction season. Red dot represents the supraorbital hump with brownish oedematous connective tissue and blue dot indicates the nuchal hump with white epaxial muscle band. Sagittal sections of the head region of female (B1) and male (B2) fish with H-E staining are also presented. The male fish possess a swollen head crest contrast to female. 1- eye; 2- retina; 3- choroid rate; 4- ocular muscle(s); 5- striated muscle; 6- skull skeleton; 7- buccal cavity

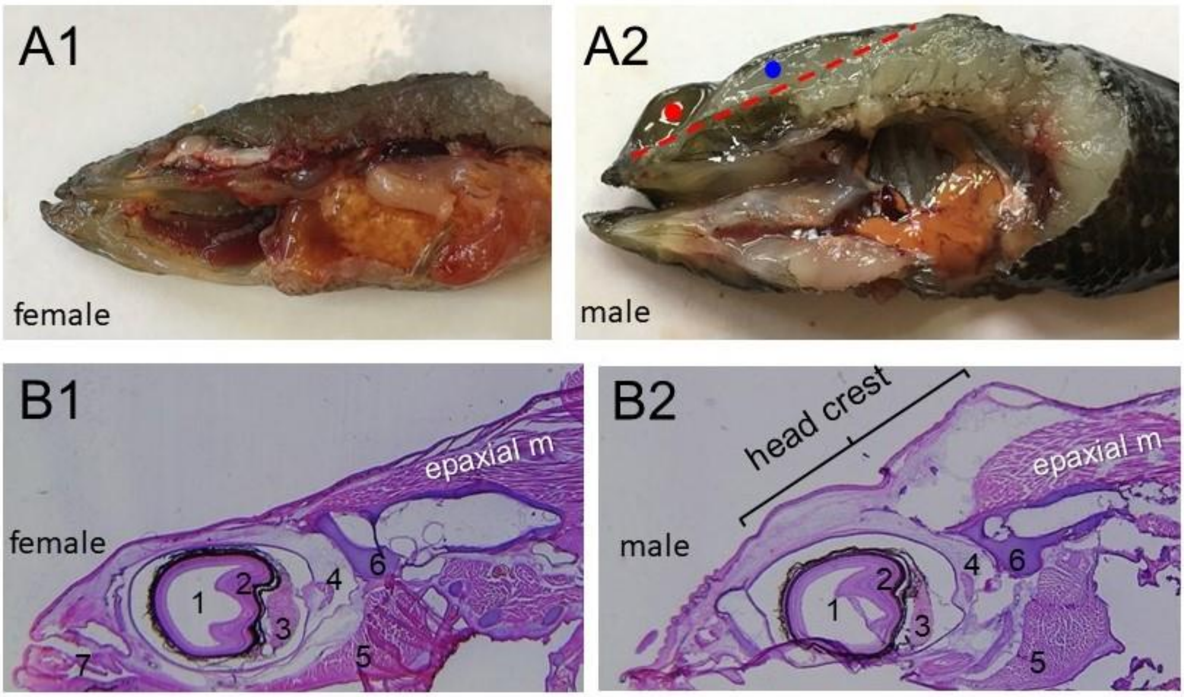
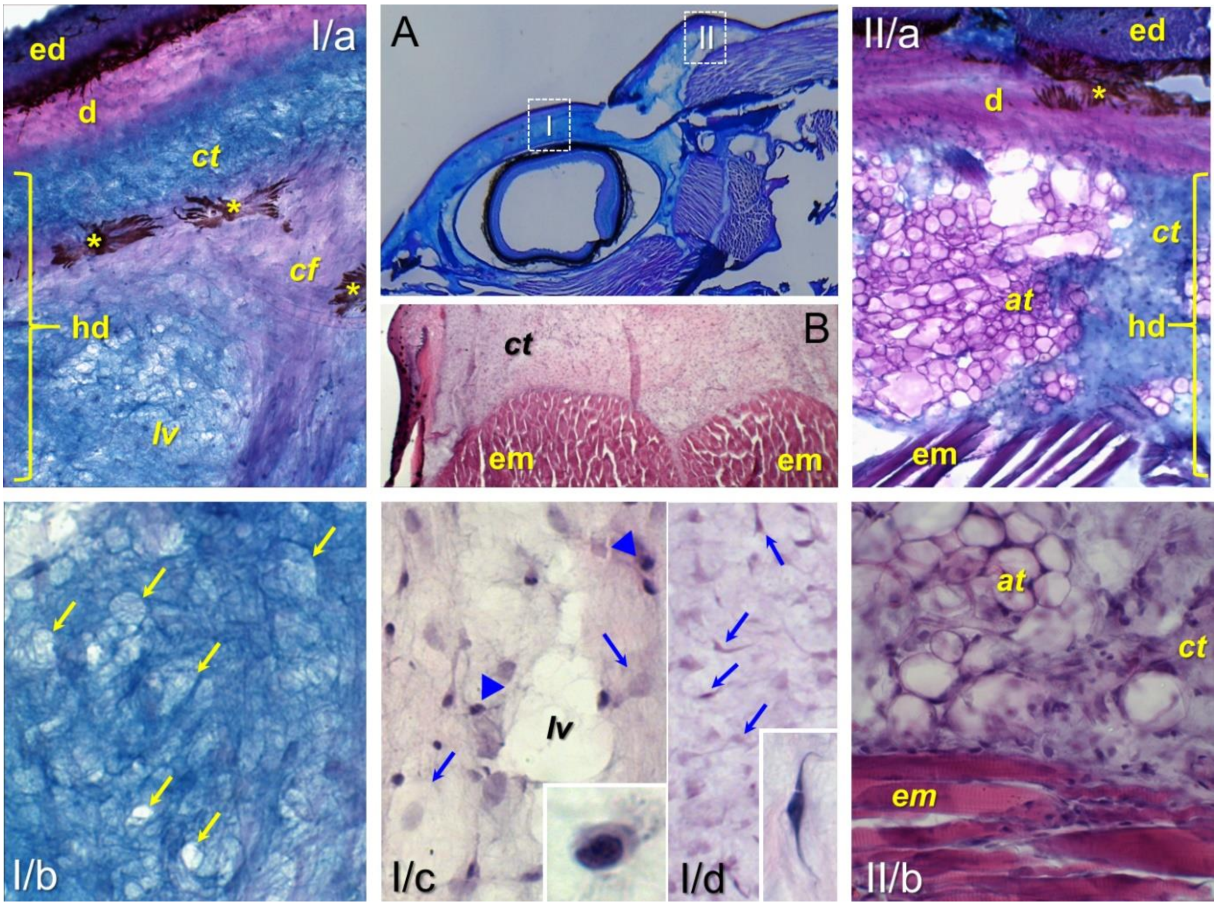


Figure 4. H-E, and alcian blue-PAS histological stainings of head crest of male Amur sleeper *Perccottus glenii* during the reproduction season. (A) Swollen head crest of male stained with PAS. On the sagittal overview image, two regions (I. – supraorbital hump, II. – nuchal hump) of the head crest were indicated by dashed squares. (B) Frontal plane of head region, H-E staining. ct- connective tissue (Wharton's jelly-like) of head crest based on hyaluronic acid, mucopolysaccharide and number of fibres produced by mesenchymal cells; em- epaxial (white) muscle, dorsal surface. (I/a) Histological organization of the supraorbital hump of the head crest, alcian blue-PAS staining, sagittal plane; ct- connective tissue (Wharton's jelly-like) with cf- collagen fibres, and lv- (empty) lipid vacuoles, furthermore, ed- epidermis, d- dermis, hd- swollen hypodermis layer, stars- melanophore cells. (I/b) Higher magnification of lipid vacuoles (lv). Arrows indicate the empty lipid vacuoles. (I/c) Permanent mesenchymal cells (triangles, see also insert) and fibroblast (arrows) are presented in this high magnification sagittal section of crest tissue. lv- empty lipid vacuoles. (I/d) Wharton's jelly-like tissue with pluripotent (embryonal) mesenchymal cells (arrows, see also insert) in the crest tissue (H-E staining, sagittal section). (II/a) An overview sagittal section of the nuchal hump of the head crest with epaxial muscle (em) after alcian blue-PAS staining. ct- connective tissue of head crest; ed- epidermis, d- dermis, hd- hypodermis; at- adipose tissue (fat cells) in hypodermis layer; star- melanophore cells. (II/b) Higher magnification picture about alcian blue-PAS stained fat cells (at) and epaxial muscle (em). In connective tissue (ct) of head crest fibroblast and mesenchymal cells are also observed around fat cells.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