but all were found to have only a single layer of prismatic calcium phosphate. The same was also true of other groups of chondrichthyans examined, namely chimaeroids and batiforms. Also, only the jaw cartilages were found to have these additional layers of prismatic calcium phosphate; all other skeletal cartilages only had a single layer of prismatic calcium phosphate surrounding them.

The fact that these multiple prismatic calcium phosphate layers are found in only some species of the families Lamnidae and Carcharhinidae leads us to the conclusion that they are not of phylogenetic importance in living chondrichthyans. Rather, because the layers are only found in the largest species of these two families, it would appear that they are a functional attribute. Perhaps, as these large carnivorous fishes grow to such a large size, the strength of their bite becomes so strong that without these additional prismatic calcium phosphate layers surrounding their jaw cartilages to strengthen them, their jaws would not be able to sustain the pressures exerted by the jaw muscles when biting.

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Evidence for spontaneous neuro-melanophore activity in *Pseudopleuronectes americanus* (Teleostei; Pleuronectiformes) during total darkness

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Summary. In total darkness, melanophores of much of the integumentary pattern of *Pseudopleuronectes americanus* display intermediate melanosome distribution, whilst the α-adrenoceptor antagonist phentolamine evokes their complete dispersion. The intermediate condition is not attained in total darkness in locally decentralized melanophores. It is proposed that, in the absence of photic stimulation, spontaneous neural activity sustains a partial melanosome aggregation in this species.
Key words. Melanophores; integumentary nerve plexus; phentolamine; spontaneous neural activity.

It is well established that albedo has aggregating and dispersing effects on melanosomes when teleosts are subjected to contrasting illuminated backgrounds, resulting in changes in integumentary hue. Such responses involve varying relative degrees of neural and hormonal regulation in different species[1,2], being predominantly neural in the pleuronectid flatfish *Pseudopleuronectes americanus*[3]. The neural control of teleost melanosome aggregation is sympathetic[4,5], with noradrenalin the neurotransmitter[6–8]. In total darkness it has been demonstrated[1,9,10–14] that several teleost species, including winter flounder[12], develop various intermediate hues. The purpose of the present work was to determine whether the intracellular distribution of flounder melanosomes in total darkness involves neural mediation, or whether it constitutes a more passive intrinsic condition of the melanophores.

Methods

Locally collected *Pseudopleuronectes americanus* (300–400 g), were initially laboratory acclimated in stock tanks. Melanophore activity in the mid region of the integumentary pattern, i.e. in the extensive general background component[3,12] was investigated. This component displays no chromatic sexual dimorphism (Burton, unpublished observations) and unsexed, postspawned and mature flounders were background adapted in individual black (B) or white (W) plexiglas aquaria (400 × 225 × 203 mm) supplied with running seawater (6.5–7.0 °C). The aquarium system was surrounded by a frame covered with a double screen of heavy duty black polyethylene arranged to eliminate extraneous illumination, whilst permitting access of personnel. Flounders were maintained under a diurnal photocycle regime (dark 9.5 h, light 14.5 h; 60 W 1 m above the fish) with

automatic time control, or under continuous total darkness. Spinal nerve section[6], performed under tricaine methane sulphonate anaesthesia[15], locally decentralized melanophores. The α-adrenoceptor antagonist phentolamine mesylate (PHA) (Ciba-Geigy) and L-noradrenalin bitartrate (NA) (Sigma) were injected subcutaneously, the vehicle being a balanced salt solution (BSS) with the following composition in mmol: NaCl, 175.0; KCl, 2.7; $MgCl_2$, 6 H_2O, 0.64; $CaCl_2$, 1.53; $NaHCO_3$, 5.0; glucose, 5.6. Doses were determined as mol/kg, with an injection volume of 0.1 ml/100 g. Control injections involved the vehicle alone. Scale slips[16] were plucked ($\leq$ 3/sample), immediately frozen (−78 °C), then fixed in formaldehyde (40 %) and mounted in glycerol for microscopic examination. Winter flounder possess morphologically distinct dermal and epidermal melanophores[17] with microscopic focal plane differences. Apparent changes in melanophore shape, associated with melanosome translocation along the processes, were recorded using the classical[18] five-stage melanophore index (1, complete melanosome aggregation; 5, complete dispersion) adapted as dermal and epidermal (DMI and EMI) scales for this species[17]. One investigator either read the scale slip melanophores, or checked the readings, for all the experiments, although subjectivity was not a critical factor with the large changes observed. During the diurnal photocycle regime, scale slips were plucked 4 h after the overnight dark phase, and at the equivalent time during continuous darkness. Procedures were facilitated in the dark by local illumination for short (sec) periods from a small pen torch, which did not evoke any observable B or W adaptive responses from the fish. Statistical analyses were performed by the Mann-Whitney U-test, using extended tables[19].

Results

During the light phase of the diurnal photocycle regime there was darkening or paling of the general background component of B (n = 10) and W (n = 10) groups of flounders respectively. Paling, which was relatively rapid, resulted in pale grey or cream hues, while dark grey or brown hues were produced by the slower darkening, these shades being reflected in the DMI and EMI values for the 2 backgrounds (table 1). After 24 h in total darkness, the general background component in these flounders was an intermediate grey or brown, regardless of background. On B, the mean DMI and EMI in total darkness were significantly lower than in the light (table 1). On W, the mean DMI and EMI increased significantly in total darkness (table 1). For flounders on B, intermediate degrees of melanosome aggregation did not occur if there was the slightest trace of low intensity extraneous light over prolonged periods.

Injection of 12 flounders, adapted to total darkness, with PHA usually evoked completion of melanosome dispersion (table 2). There were no significant changes in the intermediate melanosome distribution in the same flounders in response to BSS control injections (table 2). A further 5 flounders were subjected to spinal nerve section, resulting in prominent black bands across the general background component, and which remained dark when flounder paled on W under the diurnal photocycle regime. The extent of each band was drawn to facilitate scale slip sampling during subsequent B adaptation under the photocycle regime, when there was no significant difference between normal and decentralized melanophores (table 3). However, under continuous total darkness, melanosomes of decentralized melanophores remained completely dispersed in contrast to the intermediate condition in normal melanophores, this difference being significant (table 3). Injection of these 5 flounders with NA (3×10^{-6} mol/kg) on B under the diurnal photocycle regime evoked an even rate of paling, and complete melanosome aggregation, with no distinction between decentralized and normal melanophores. Thus, the circulation of plasma-borne stimulants to the decentralized melanophores was not impaired by the surgery. In each experiment there were similar trends in DMI and EMI values.

Table 1. Comparison of melanophores under diurnal photocycle regime and subsequent continuous total darkness

	Diurnal photocycle (24 h)	Continuous darkness (24 h)	P
B background			
DMI	4.8 ± 0.1	3.3 ± 0.1	< 0.001
EMI	5.0	3.1 ± 0.2	< 0.001
W background			
DMI	1.7 ± 0.2	2.8 ± 0.1	< 0.001
EMI	1.2 ± 0.1	2.5 ± 0.3	< 0.001

Melanophore index values = mean ± SEM; n = 10 flounders on each background.

Table 2. Effect of PHA injection (2.5×10^{-5} mol/kg) on the DMI under continuous total darkness

Background	BSS injection		PHA injection	
	DMI before	DMI 1 h after	DMI before	DMI 1 h after
Black	3.5 ± 0.3	3.4 ± 0.4*	3.4 ± 0.2	4.8 ± 0.2**
White	2.8 ± 0.2	3.0 ± 0.3*	2.9 ± 0.2	5.0**

DMI values = mean ± SEM; n = 6 flounders on each background. *p > 0.1, **p < 0.005.

Table 3. Comparison of DMI of normal and decentralized melanophores of flounders on B under diurnal photocycle regime and subsequent continuous total darkness

Regime	Normal DMI	Decentralized DMI	P
Photocycle	4.9 ± 0.1	5.0	> 0.1
Darkness	3.5 ± 0.2	5.0	< 0.005

DMI values = mean ± SEM; n = 5 flounders.

Discussion

Although control of physiological responses of flounder melanophores is predominantly neural[3], these responses are slower than in many teleosts. Equilibration, particularly on W, requires up to a week under both continuous illumination and repeated diurnal photocycles[20]. However, the intermediate condition in flounder after 24 h total darkness was comparable with observations on other teleosts[9,10,11,14], and the observations represent the first cellular analysis of the phenomenon in this species. The control of teleost melanophores in darkness is obscure. Mechanisms which have been suggested, without experimental support, include a persistent neural influence[21,22] and interaction between melatonin, melanocyte stimulating hormone (MSH) and melanocyte concentrating hormone (MCH)[20,23,24]. However, neural mediation has been disputed[1] since equilibration times in total darkness are protracted in most species. In contrast with other species[25–27], the pituitary of flounder is unnecessary for completion of melanosome dispersion[3], and the plasma MCH is inadequate to aggregate melanosomes during prolonged (days) exposure of flounder to W after spinal section. Although flounder decentralized melanophores are refractory in total darkness they respond to plasma-borne injection. Thus, the normal intermediate condition in the absence of photic stimulation cannot be solely hormonally evoked. However, such a conclusion does not preclude a possible hormonal influence in melanosome dispersion after decentralization, and antagonism between such a hormonal influence and NA in the intermediate condition.

Interpretation of data from injected plasma-borne neural inhibitors can be complex due to the potential multiplicity of regulatory levels which can be antagonized. For PHA, such levels include melanophore α-adrenoceptors in teleost in vitro preparations[7,28–30] in which this antagonist evokes melanosome dispersion. The current results with PHA, and with local decentralization, provide compelling evidence that in flounder the melanophore condition in total darkness involves neural activity releasing NA from the integumentary neurons. The effect of local decentralization also suggests that this spontaneous neural activity is not initiated peripherally in the spinal nerves or integumentary nerve plexus, but is more central in origin. Photic stimulation would thus modulate such spontaneous neural activity thereby increasing melanosome aggregation or dispersion depending on the albedo conditions. Melanophore activity associated with the absence of photic stimulation should provide an interesting comparison for further studies involving red and other monochromatic light.

It has been suggested that the melanophore conditions in darkness[31] and on illuminated B[32] both represent a passive state, but it is not clear whether there is any bioenergetic basis for the concept of a resting state for these effectors. However, it is clear that in this species the intermediate condition of melanophores in total darkness is associated with spontaneous neural activity, and is not a passive intrinsic property of the effector.

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