Squids old and young: Scale-free design for a simple billboard

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ABSTRACT

Squids employ a large range of brightness-contrast spatial frequencies in their camouflage and signalling displays.

The 'billboard' of coloured elements ('spots' = chromatophore organs) in the skin is built autopoietically–probably by lateral inhibitory processes–and enlarges as much as 10,000-fold during development. The resulting two-dimensional array is a fractal-like colour/size hierarchy lying in several layers of a multilayered network. Dynamic control of the array by muscles and nerves produces patterns that recall 'half-tone' processing (cf. ink-jet printer). In the more sophisticated (loliginid) squids, patterns also combine 'continuous tones' (cf. dye-sublimation printer).

Physiologists and engineers can exploit the natural colour-coding of the integument to understand nerve and muscle system dynamics, examined here at the level of the ensemble. Integrative functions of the whole (H) are analysed in terms of the power spectrum within and between ensembles and of spontaneous waves travelling through the billboard.

Video material may be obtained from the author at the above address.

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1. Introduction

There are now many descriptions of the camouflage, signalling and warning "colours" displayed by cephalopod molluscs [1,2]–alongside decades-old findings on the anatomy, physiology and pharmacology of brain, nerve and muscle thought to be behind these dynamic events [3–5]. However, attempts to match cell-level analyses with the behavioural diversity encountered in the field and in captivity can be a rather frustrating business. Laboratory findings concentrating on a single 'species' of chromatophore organ have tended to be generalised, and non-responders are often overlooked; little is known about the pharmacology of small yellow tegumental chromatophore organs lying in the most superficial layer of the skin of squids, even though this is the most numerous class; details of pattern expression have often been assumed to be exclusively under nervous control—which is far from the case. This article shows how the gap between the whole animal and the cell level can be bridged by *photographic images and video-recordings of colour change acquired under semi-natural conditions* at magnifications sufficient to map 'molecular' information onto 'molar' (Fig. 1). As long as the events recorded are spontaneously generated by the animal within its normal physiological range, they can be assumed to be a true record of the natural system.

This (reverse) engineering approach to the workings of the billboard and how it is built has enormous heuristic potential—and is simple. But there are unwritten terms for any success: a degree of *cooperation with the living subject* unfamiliar to investigators habituated to one-way control over experimental conditions.

2. Whole animal recording

At the low end of our magnification range is this remarkable photograph of the Caribbean Reef squid (Fig. 2). Though free swimming in the open sea, details are sufficient to make out the basic elements that contribute to *brightness-contrast information*. Individual chromatophore organs (1)–*spots* for short, since it is the pigment spots that supply the 'ink' of the billboard–are discernible where differentially expanded to create the dark *feature* spanning the middle of the body. Totally inactive spots are at or below the limit of resolution. Muscles and nerves which modulate differential expansion of spots in the plane of view remain invisible. Other kinds of structure contributing to pattern generation–specular (mirror) reflectors on and around the eye (2), broad-band scattering (white) spots (3), layers of iridocytes producing greens by interference (4)–are indicated in the same figure.

2.1. Sustained patterns

More than 40 features such as the bar just mentioned have been documented for *Loligo vulgaris*. All of them involve groups of chromatophore organs (*ensembles*) acting together. Fished in the Bay of Naples, this species survives well in confinement—especially in the company of conspecifics. Many of the fixed-action
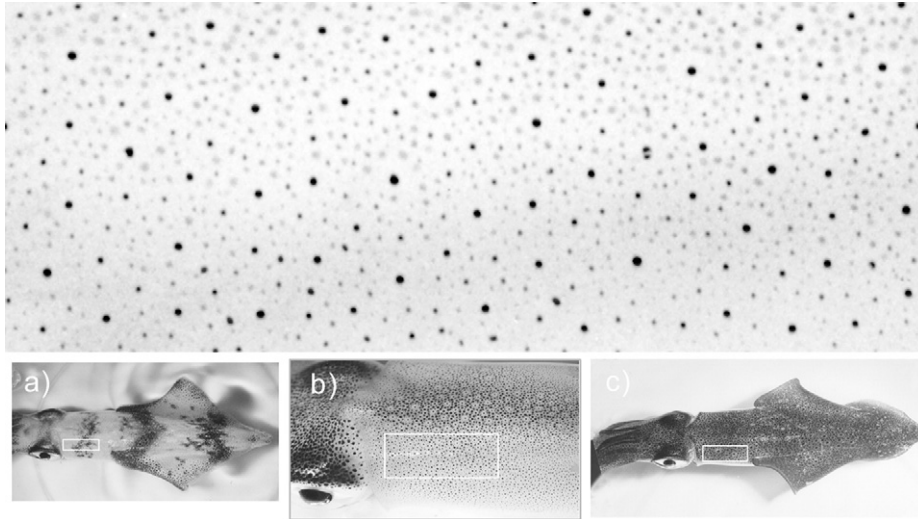
E-mail address: andrew@packards.de

Fig. 1. Adult squid (*Loligo vulgaris*, ♂ no. LV0694). *Above*, detail of the 'billboard', animal 'pale'. Image width c. 1.5 cm. *Below*: three camouflage patterns generated by this billboard: (a) 'cryptic', (b) 'counter-shaded' (see Fig. 6), and (c) 'all-dark'. (White rectangles show location of the detail above.)
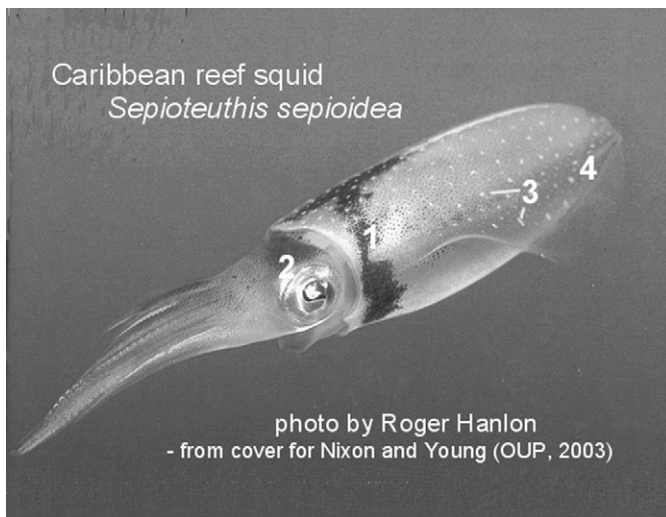

Caribbean reef squid
Sepioteuthis sepioidea

photo by Roger Hanlon
- from cover for Nixon and Young (OUP, 2003)

Fig. 2. (See text).

patterns continue to exhibit when individuals are isolated in bowls used for photography; some may be worn for considerable periods of time. Amplitude varies (see Section 2.2).

2.2. Transient colour changes

The contents of Box 1–towards the higher end of our magnification range–are from a video-recording of transient "colour" changes on the flank of a restrained, but otherwise normal squid (*L. vulgaris*). All frames are of the same spots. At this magnification, the details of the 'billboard' are sufficient to define the size, "colour", relative position and other properties of the elements (spots) composing it as well as scoring their contribution to different phases of the recorded changes (see Box 1, text). (Figures on frames are the running clock).

Fig. 3, from the same video, analyses the contribution of individual spots to three phases of darkening through neuromuscular expansion.

From such data one can link directly to the distinctive processes evoking the activities. The main key to dynamics turns out to be a developmental one.

3. How is the billboard built, and what is the significance of the process?

3.1. Development

Squids grow continuously. For purposes of classification, the diagnostic characteristic of chromatophore ensembles in any one part of the skin, at any one stage, is the *resting size of pigment spot relative* to others in the neighbourhood. At hatching (Box 2) *L. vulgaris* possesses a few (c. 100) well-spaced spots on arms and head and mantle, seen here in the retracted condition (ventral view, note conspicuous eyes and ink gland). The much smaller paralarva of the oegopsid squid *Dosidicus*–which hatches 7 days after fertilisation–has less than 20; 9 are seen here (dorsal view) both in the retracted and expanded states [6].

As the billboard enlarges with growth of the animal–ultimately many 1000-fold in area–interdigitation of new elements maintains the regular-irregular spatial arrangement of spots, with extant spots determining the placing of all future spots. The process of recruitment from germinal layer(s) deep in the integument can be accounted for by Meinhardt and Gierer's theory of *lateral inhibition* governing the formation of other biological patterns [7]. One effect is that new spots are smaller than pre-existing ones, as well as more closely spaced, and a size hierarchy quickly becomes established. As spots do not usually die or disappear at later growth stages, the process can be read backwards in time.

The histogram of numbers against resting size of red and brown pigment spots given in Box 1 reveals a power-law distribution: largest spots are the fewest and earliest to arise, smallest spots are the most numerous class and latest to arise. Yellow spots in the same frame (see text)–too small to be measured in the baseline condition–form the largest of all classes and confirm the histogram's power-law distribution.

Fig. 4 shows the first frame in Box 1 at a resolution sufficient to see all size-classes. The central 'nuclear' spot (**A** in Box 1) lies deepest in the skin and belongs to the earliest (largest) size-class.

Box 1–Squid colour change dissected.

Above: c. 3.5 × 5 mm video-clip details of the display surface ('billboard') of an adult *Loligo vulgaris* during natural ''colour change''.

Middle: four phases from the same video-clip. Each frame contains 300 yellow chromatophores (pale spots) of two size classes (≈70% of total), and ∼130 brown (B) and red (R) spots of several classes (≈30% of total): **A** and **B** reference spots. a) baseline condition for all ensembles, the skin is 'white' (pigment spots cover <3% of display surface); active retraction of all classes is a rebound condition (score 0) following a burst of maximum excitation of surrounding muscle (indicated for one spot). b), c) 'all-dark'; c) through maximum expansion of B and R (score 1) and contribution from yellow; b) with minimum contribution from yellow. d) differential pattern (B and R classes only).

Bottom: thresholded spots of upper left frame (B and R only) analyzed for numbers and relative size at rest (*x* axis of histogram) (ImageJ).

count 118
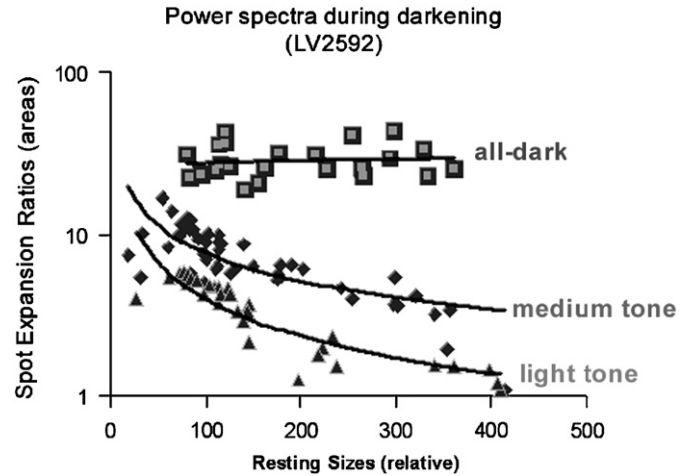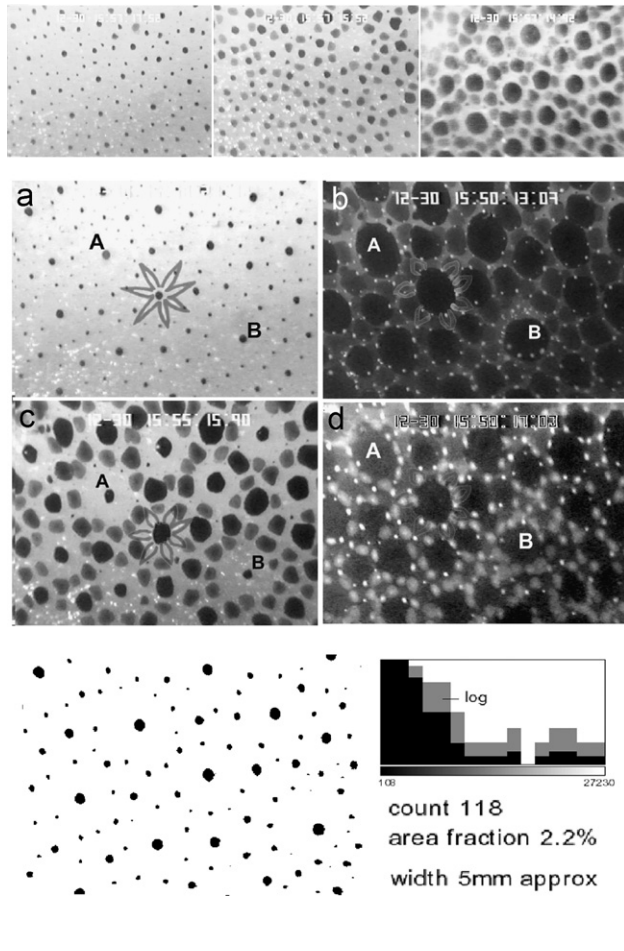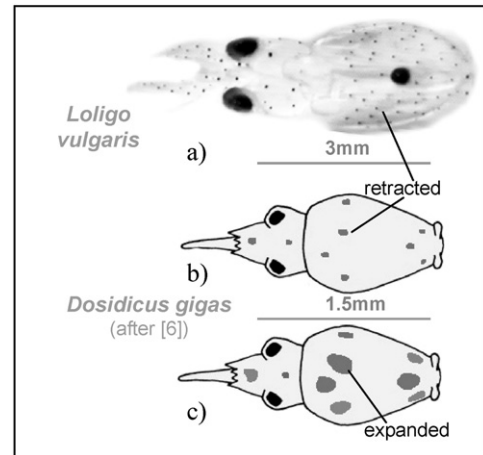area fraction 2.2%
width 5mm approx

Fig. 3.

Fig. 3. Logarithmic increase in power distribution amongst red/brown chromatophore size classes during 'light', 'medium' and 'all-dark' phases of natural 'colour change': (LV2592, same image sequence as Box 1). Semi-log plots. Least-squares trendlines, $y = cx^b$. (N.B., relative paucity of measurements in 'all-dark' is due to masking of spot outlines during this phase.)

Box 2–. Spots on squid hatchlings.

Linked members of two of the smallest classes ('5' and '7') are shown, with no links between classes. A similar detail of thresholded pigment spots is shown below right (only one 'yellow' class indicated) with key to the size/colour hierarchy alongside (left).

3.2. Colour

Spots are pale yellow or orange at birth, and rapidly darken as their ommochrome pigments polymerize. The darkening process (which in *Octopus* is accompanied by accumulation of nickel) never quite catches up with that of previous generations, so that a scale of colours (Fig. 5) established which correlates with the main measure (relative size of spot). Combinations of hue and of optical density, easily appreciated by the eye, vary with state of expansion of chromatophores; they have recently been examined from the materials science point of view [8,9].

The several levels of the size/colour/age hierarchy (corresponding to generations) lie in successive layers of the skin, from inwards (oldest) to outwards (youngest).

4. Differential activity of spots

Fig. 6 analyses the contribution that the spots of any ensemble make to three only of the patterns displayed in captivity by one particular squid. (This is the same animal as Fig. 1. Detailed views
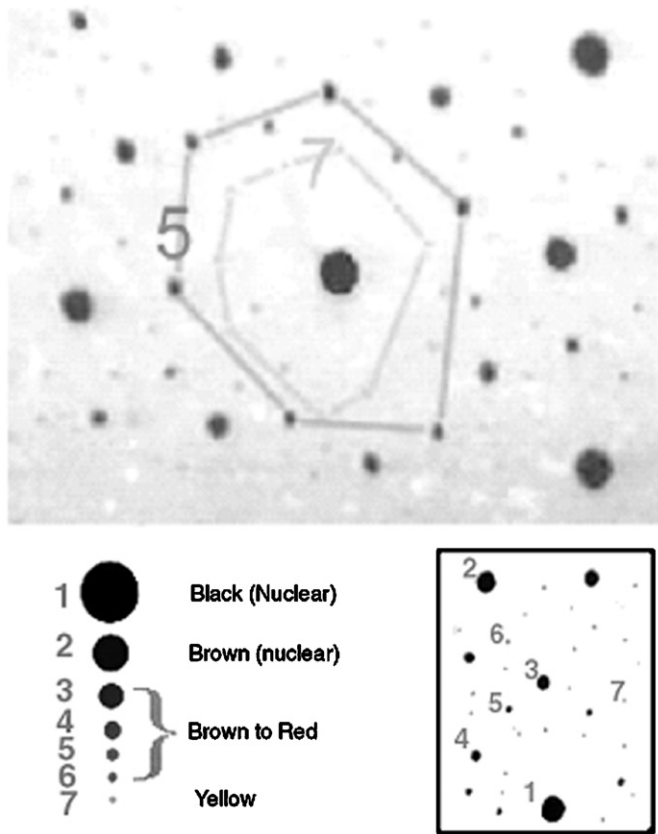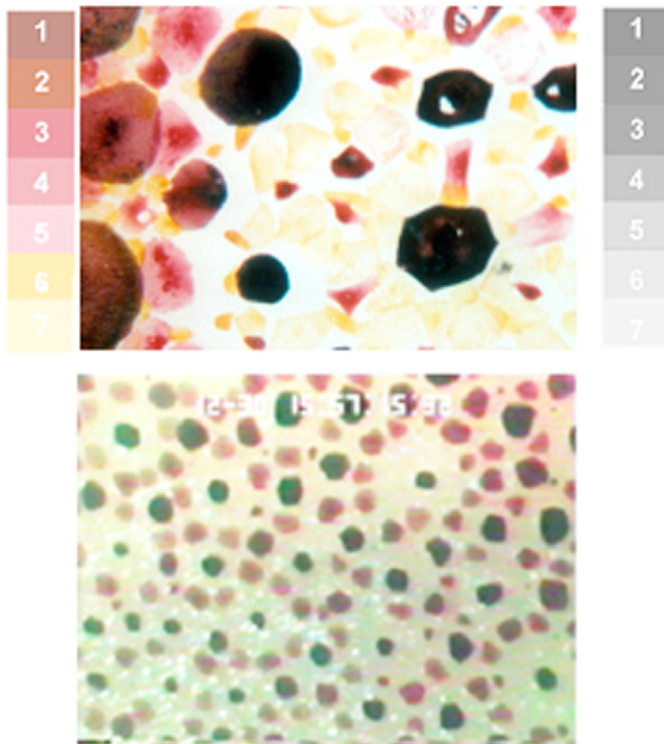
Fig. 4. (See text).

Fig. 5. *Colour, tone and shapes of chromatophores.* Scales (1–7) of colour (left) and of tone (right) sampled from photograph of tonically expanded spots of an adult *Loligo vulgaris* mantle. Different shapes are due to differential contracture of some only of total radial muscle fibres subtended by spots following post-mortem treatment with salicylic acid.

are of the left side of the mantle, in dorso-lateral view: i.e. the same region of the billboard as in Box 1.)

4.1. Activity gradients

In the first (Fig. 6a), the squid is pale yellow above. Spots of the two yellow classes ('6' and '7') are partially expanded, amplitude (degree of expansion) is graded dorso-ventrally; their profiles are circular, and the two classes are of slightly different tone. All brown and red classes ('1' to '5') are in the *baseline* condition (fully retracted = zero amplitude); in this state they cover only 2% of the viewing plane.

In the second (Fig. 6b), the squid is 'counter-shaded'; darkening activity by brown and red spots is graded dorso-ventrally.

4.2. The differential contribution

The differential contribution that red and brown ensembles make to Fig. 6b is shown in Fig. 6c, which plots the expanded size of participating spots against their resting (baseline) size (measured in 'all pale', Fig. 6a). The resulting relative amplitude curve shows spot activity as inversely related to resting size ('age'): i.e. *xy* is constant. In this characteristic pattern, all spot classes are generating the same spatial frequency. In Fig. 6d and e ('disruptive'), the trend amongst spots creating the cryptic bar is broken by some of the older spot classes (ringed).

There is also an inverse relationship to resting size in the distribution of power amongst spots supplied by a single unit (Fig. 7); none of them is maximally expanded.

Such data can be used to work out the patterns of connections of nerves supplying the skin and the distribution of power in the central pattern generators (see Section 6).

4.3. Spot profiles

Differential activity of muscles *within* an ensemble–and thus of the nerves supplying any chromatophore organ–is indicated by the shapes of partly expanded spots (Fig. 5). Polygonal profiles are a sign that some only of a chromatophore's radial muscle fibres are contracting (see Fig. 8). Circular or oval profiles, by contrast, are the result of all (c. 25) fibres contracting in unison. (Easily picked up by the eye, this differential activity has not been analysed.)

4.4. Oceanic squids

The billboard of oegopsid squids (Fig. 9) is much simpler than that of loliginids. The same size hierarchy exists but the millions of spots of a full grown Humboldt squid (*Dosidicus gigas*) are all of one colour–seen here being stimulated electrically. The most conspicuous 'colour pattern' of this species is a temporal one of white flashes–sometimes in a duet with a neighbouring squid [10]. Flashing dark-to-light means synchronous relaxation of contracted chromatophore muscle over the entire surface of the body; central processes are unknown but peripheral processes presumably involve hyperpolarisation of muscle.

5. Automaticity

When chromatophore ensembles are free or have been artificially freed from nervous control, they exhibit spontaneous (autonomous) activity. In the squid *L. vulgaris*, such activity is seen most dramatically as repetitive waves of colour which travel across the skin at c. 1 cm s^{-1}—revealing that spots are coupled.
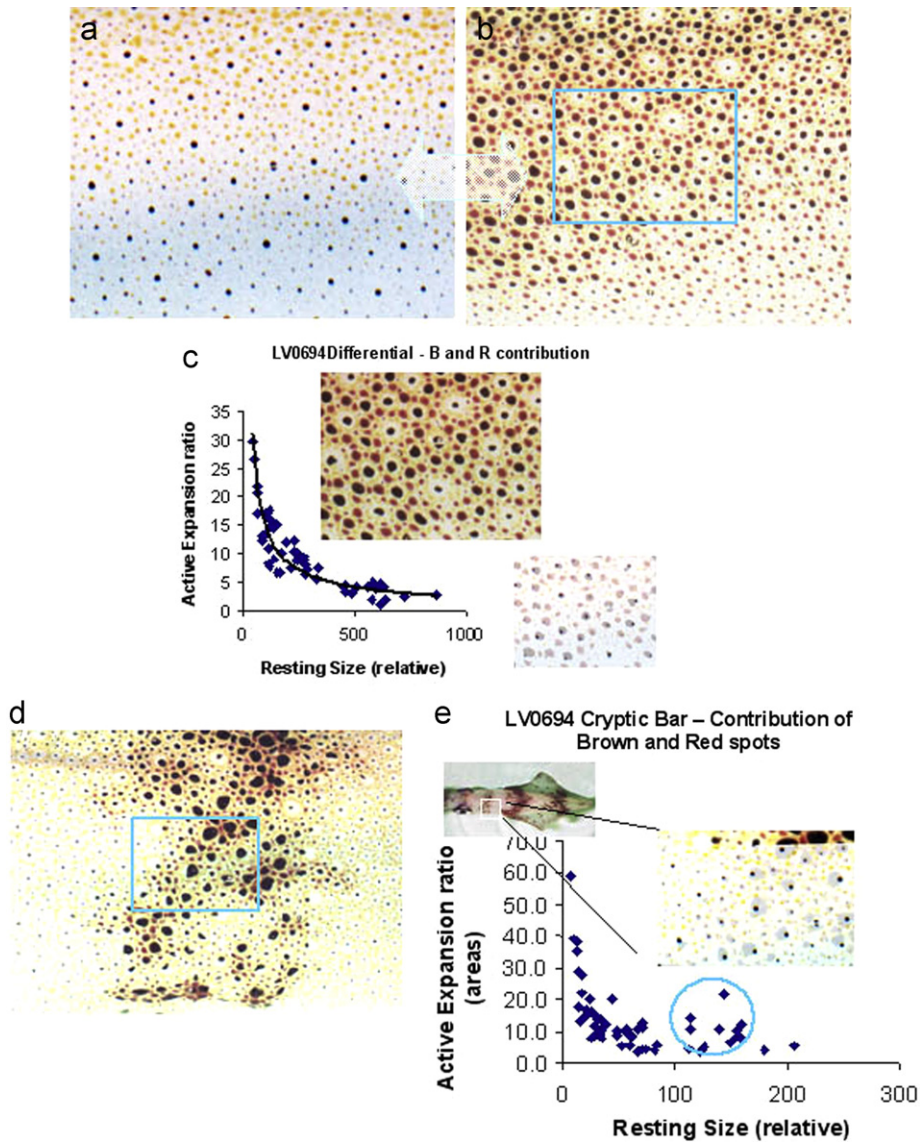
Fig. 6. *Power distribution*: close-ups of the mantle of *Loligo vulgaris* (LV0694) as in Fig. 1 (detail); (a) *pale*: all brown/red spots in baseline condition, two yellow classes (partially expanded) show dorso-ventrally graded activity; (b) *counter-shaded*; (c) differential contribution of brown/red size classes to the detail indicated in (b); (d) *cryptic* bar; (e) differential contribution of brown/red size classes to detail indicated in (d): larger resting size classes ('2' and '3') ringed. (Least squares trendlines, $y = cx^b$.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

5.1. Fast waves (FW)

Fig. 10 analyses the participation of selected brown, red and yellow spots in one such sequence of fast colour waves. Activity is seen building up on the poorly innervated ventral surface of a recently dead squid, first on the intact (right) side then crossing to the left side where it self-reexcites, amplifies, and entrains the numerous yellow spots. The left side of the squid had been surgically deprived of innervation (denervated) several days earlier.

'Horizontal' control of the network(s) engaged in automaticity [11] may have just as important role in normal pattern generation as 'central' control, but the mechanism of their interaction is not known (see Section 6).

5.2. Wave power

Fig. 11a is a frame from a video-recording of another unilaterally denervated squid; two fast dark waves (FW$_D$) have travelled up from the ventral to the dorsal surface on the

denervated side and collided before becoming extinguished as they hit the midline area bounding the intact (innervated) side. The part played by the different size/age classes of brown and red spots in this long sequence of waves is seen in Fig. 10b: the larger the resting size of the spots (i.e. the older the size/age class) the greater the relative amplitude of their transient contribution to the propagating wave front. The trend is of opposite sign to that in Fig. 6c.

6. Discussion

How well do these simple measurements of transient and sustained activity amongst chromatophore ensembles agree with knowledge obtained by more conventional, invasive, means? What does the reverse engineering approach tell us about the way a living squid generates information targeted at eyes, and is the 'print out' comparable with such techniques as the half-tone process?

It is known from standard behavioural, neurophysiological and surgical investigations, etc. (for references see [4,5]) that the displays presented on the billboard are drawn from a limited repertoire of fixed-action patterns coded in the circuitry of a complex brain. They can be evoked by electrical stimulation of its higher motor centres, without specific input from the squid's eyes or other sense organs. Common output is through motor neurones in lower brain centres. Variations in amplitude–to match the reflectance of the background, for instance–are directly related to frequency of firing of motor units (i.e. a motor neurone and its peripheral connections). Increases in firing frequency produce larger responses (a) by summating contractions, (b) by spatially extending the response, and (c) by recruiting spots of progressively larger resting size. Combinations of excitation and of inhibition of motor units generate brightness-contrast of various spatial frequencies resulting in patterns of different shape and form–much as in other motor control systems. (Contraction/relaxation times of muscle altering the sizes of spots depend on its mechanical properties and, even for rapid changes, are orders of magnitude longer than the duration of a single nerve impulse.)
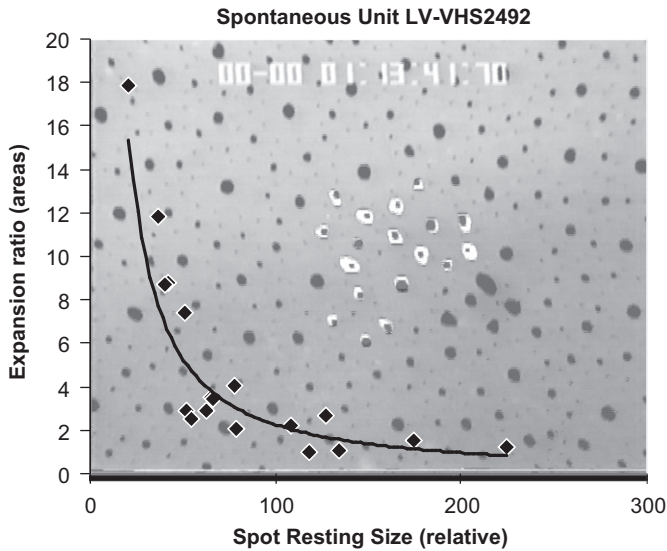

Fig. 7. *Power distribution within a single unit*: relative power distribution amongst 18 chromatophores of different size/age class belonging to a spontaneously firing motor unit (*Loligo vulgaris* LV2592: dorsal mantle). Spots partially expanded when the unit is 'on' are the light profiles in the image, shown against their resting size (black) when 'off'; all other spots 'off' (a few unretracted in the baseline condition).
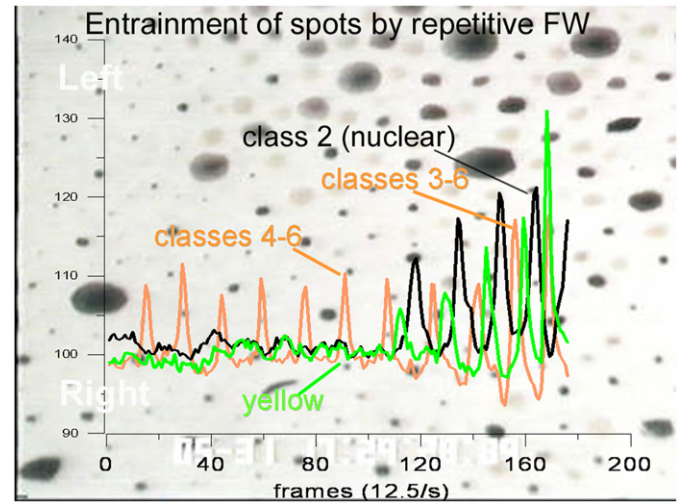

Fig. 10. Individual responses of several classes of spots during amplifying fast-wave activity (frequency c. 0.8 Hz); pre-treatment with CO_2-saturated seawater, has rendered muscle membranes unstable. (*Loligo vulgaris*, postmortem, ventral surface: left side denervated. (See text.)
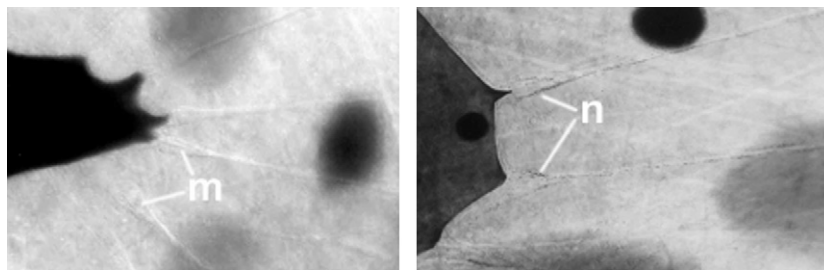

Fig. 8. Details of living chromatophore organs showing dark pigment spots partially expanded by radial muscles (**m**) and their accompanying nerves (**n**) dyed blue. (*Loligo vulgaris*, low-power interference microscope.)
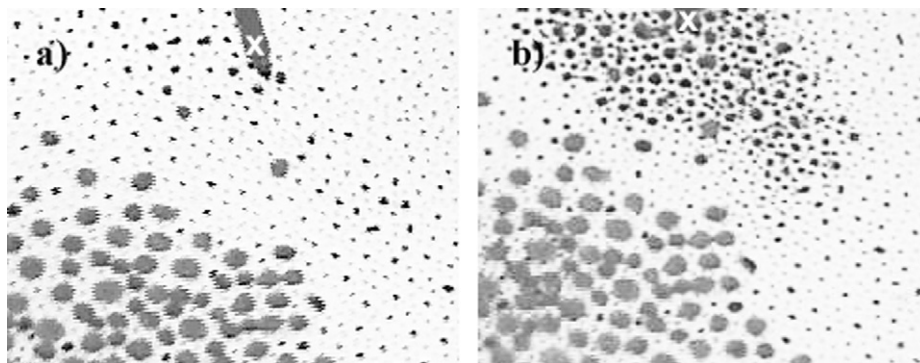

Fig. 9. Detail of the fin of the squid *Dosidicus gigas* seen from above with depolarising electrode in place (**x**), (a) before stimulation, (b) during stimulation. Stimulated expansion (darkening) of small spot classes spreads short distances as a wave. (Image width c. 1.5 cm.)
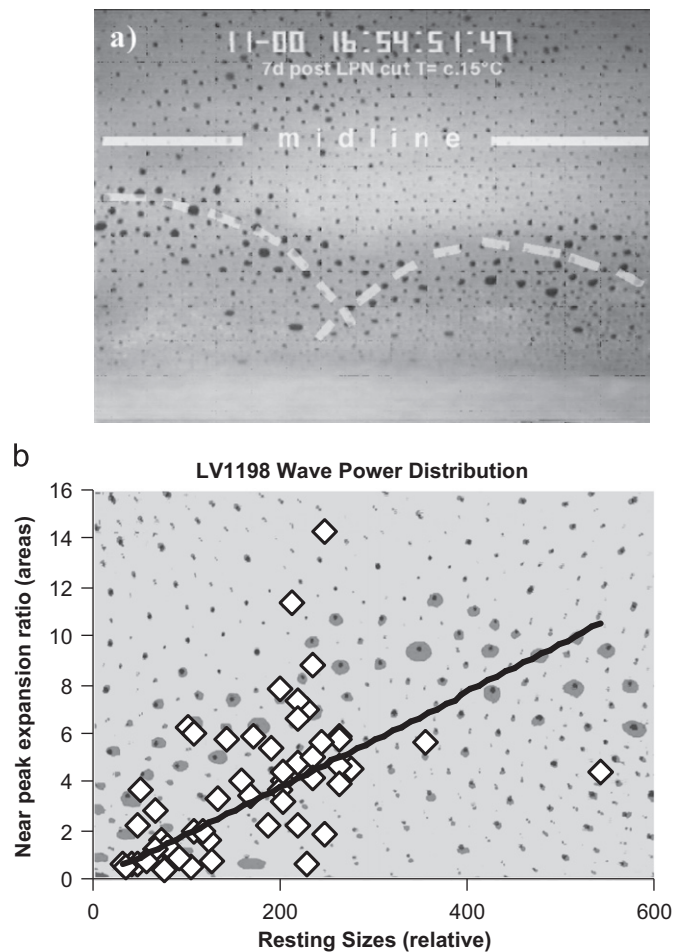
Fig. 11. *Fast waves* (FW_D). Spontaneous fast waves of colour (brown/red spots only) travelling at c. 1 cm s^{-1} across the left flank of a squid deprived of central nervous input (*Loligo vulgaris*, LV1198). (a) See text; (b) relative expansion ($\diamond$) of individual spots at peak expansion in the crest of a wave (red/pale), against resting size (black).

Connectivity: The adoption of a single dimension (a spot's resting versus expanded size) to measure natural patterning in the whole animal has brought to light several interesting correlations. The finding that differential activity of chromatophores (spots) is a function of size-class at rest is also the key to connectivity—in understanding how the wiring of the billboard is established during development. Unlike its octopus relatives, recruitment of new spots into the skin of a squid as it grows has not been followed directly; nevertheless, as with the young octopus [12], counts of spot sizes on macro-photographs of squids taken at any stage of growth always show numbers increasing logarithmically with decreasing spot size. So the hierarchy is interpreted as a hierarchy of relative ages up to that point.

The patterns of connections that nerve fibres make at the periphery would then be, as with classical motor units, the result of anatomical/developmental constraints set by the end organs themselves: each size/age class (generation) of spots as it arises attracting connections from its own size/age class of ingrowing motor neurone.

Chromatophores are also known from electrophysiological stimulation to be multiply innervated; that is, the several muscle fibres of a given spot do not all belong to the same motor unit. When only one is firing (Fig. 7) a spot's maximum expansion is not achievable. Multiple units innervating a given ensemble may either belong to the same generation or to different generations.

The findings reported above suggest that the latter are in inverse proportion to distance in age.

The resulting overlap in wiring provides considerable variety to the potential output of the billboard; in dorsal regions of the skin (better illuminated and more visible to an observer than ventral regions), a motor unit may connect only to a few spots (see Fig. 7), and thus deliver finer spatial detail than the more extensive units found ventrally.

The skin seems to be engineered to combine central and distributed processing, but little or no work has been done to prove or refute this idea. Central drive activates chromatophore muscle frequency-dependently and keeps it relaxed (? hyperpolarised) when nerves are not firing. Following removal of central drive–which can be mimicked by removal of nerves–the 'billboard' exhibits enhanced excitability including transient reversing potentials amongst chromatophore muscle that propagate through the network(s) linking an ensemble (Figs. 10 and 11).

Since fast propagating waves of colour are also part of the natural repertoire of some squids, the question arises whether the mechanism is the same. Access to the reserve of excitability available in the older coupled ensembles (Fig. 11)–still silently suppressed by nerves at low frequencies–could be through an interstitial network [13].

I have drawn attention elsewhere [4] to the similarity of the 'billboard' to a half-tone screen. In photographic half-tone processing, grey-level variations over a given area are achieved by variations in size but not the density of individual spots; this holds for the darker (older) classes of chromatophore pigment spots, retracted to a sphere at rest, and changing little in density as they expand; but it does not hold for the younger red and yellow classes which offer a range of tones in the expanded condition (see Fig. 6). Theoretically–as advances in printer technology demonstrate–the potential grey-level 'refinement' of a billboard combining continuous-tone with half-tone processing is two or three times that obtainable with the half-tone process alone.

Acknowledgements

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