

## Cephalopod research and bioactive substances

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Marine environment comprises complex ecosystems and many of the organisms are known to possess bioactive components as a common means of self-defense or for the protection of eggs and embryos. In recent years, many bioactive compounds have been extracted, characterized and purified from various marine animals like bacteria, algae, dinoflagellates, tunicates, sponges, soft corals, bryozoans, cephalopods, and echinoderms. Present review consists of the research work done on the biology of the cephalopods, mainly pertaining to the feeding strategies (the salivary gland toxins, body and liver oils), the reproductive strategies (the ovarian-peptides, the nidamental gland products, accessory nidamental gland products and the associated symbiotic bacteria), and the defence mechanisms (the ink glands and their bioactive products, the squid-*vibrio* association, the camouflage colouration mechanisms and the reflectin-proteins). The learning capabilities and personalities of octopods have been a matter of great interest in cephalopod ethology. The aspect of cephalopod welfare in laboratory and field studies merits scientific debate because of the biological and behavioural complexities exhibited by these highly evolved, lovable invertebrates.

**[Key Words:** cuttlefish, squid, octopus, nidamental glands, symbiotic bacteria, salivary toxins, ink-peptidoglycan, reflectin-proteins]

### Introduction

The marine environment comprises complex ecosystems and many of the organisms are known to possess bioactive components as a common means of self-defense or for the protection of eggs and embryos. Some organisms derive the chemistry from dietary sources, while others synthesise the compounds *de novo*. Some compounds may be produced by associated organisms, while others may require an association between the host and microorganisms to produce the compounds<sup>1</sup>. In recent years, many bioactive compounds have been extracted, characterized and purified from various marine animals like bacteria, algae, dinoflagellates, tunicates, sponges, soft corals, bryozoans, cephalopods, and echinoderms<sup>2,3</sup>.

The word *Mollusca* comes from the Latin word *mollus*, meaning 'soft'. The phylum Mollusca includes animals that are usually soft-bodied but have hard external shells of calcium carbonate. Some molluscs like the cephalopods have evolved to having reduced, internalized shells, or to entirely losing their

shells. The class *Cephalopoda* (Gr. *Kephale* =head and *pod* = foot) includes squids, cuttlefishes, octopuses and nautilus. The subclass Nautiloidea has external shells, while in the Coeleoidea, the shell is either considerably internal or has been completely lost<sup>4</sup>. The Coeleoidea includes squids, cuttlefishes, octopods and vampire squids, which are represented by around 700 species (Fig. 1).

Cephalopods occur in all marine habitats of the world like benthic-cryptic or burrowing in coral reefs, grass flats, sand, mud and rocks; epibenthic; and pelagic in bays, seas and in the open ocean. They are found at depths ranging from the surface to over 5000 m. Many species of oceanic cephalopods undergo vertical migration, where in they occur at depths of about 400 to 800 m during the day, then ascend into the uppermost 200 m or so during the night. Shallow living cephalopods are able to conceal themselves by chromatophore-produced colour patterns and chameleon like colour changes, while many deep-sea forms camouflage themselves by producing bioluminescent light from photophores. Cephalopods are famous for their defences, from their fast jetting escape movements to changes in colouration that can

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Fig. 1—Cephalopods  
 (a) Pharaoh's cuttlefish (source: Sherief *et al.*<sup>131</sup>)  
 (b) Long barrel squid (Nair J R- personal collection)

be cryptic, disruptive or startling, to arm autotomy, to toxin venom and to inking<sup>5,6</sup>.

Among bilaterian invertebrates, cephalopod mollusks have a central nervous system that rivals in complexity that of the phylogenetically distant vertebrates. However this prime example of convergent evolution has rarely been the subject of recent developmental and evolutionary studies, which may be partly due to the lack of suitable neural markers and the large size of cephalopod brain<sup>7</sup>. The

giant squid's body is of great length and it uses a single large nerve cell to send the escape message from its brain to its lower body. In 1963, the British Scientists Alan Lloyd Hodgkin and Andrew Fielding Huxley won the Nobel Prize (Physiology or Medicine) for their description of the behaviour of nerve impulses, which was based on the Atlantic squid (*Loligo pealei*) giant axon – a neurological model that has no peer in the animal kingdom. Major part of the work was carried out at the Marine Biological Laboratory, Plymouth. In fact much of the basic knowledge of the mechanism of nerve fibre or axon impulse conduction has been obtained from the squid giant axon<sup>8</sup>. In the Gilbert *et al.*<sup>9</sup> edited text on '*Squid as experimental animals*', there are major chapters on the electrophysiology and biophysics of the squid giant axon; on the structure and function of the squid eye; and on the development of the squid visual system. Lee *et al.*<sup>10</sup> cultured and reared *Sepioteuthis lessoniana* through multiple generations to provide squids, especially their giant axons, for biomedical research in the USA. The elaborate study of the brain and lives of cephalopods by Nixon and Young<sup>11</sup> provides an insight into the world of behaviour of these wonderful creatures. The learning capabilities and personalities of octopods have been a matter of great interest in cephalopod ethology<sup>12-16</sup>.

### Feeding strategies

Cephalopods play an important role in the trophic web of the marine ecosystems<sup>17</sup>. They have been among the dominant large predators in the ocean at various times in geological history. Extant cephalopods are active marine predators that prey upon shrimps and crabs and other molluscs like bivalves and gastropods, and fishes<sup>18</sup>. Cephalopods are built for speed and use their tentacles and oral arms for prey capture. Cephalopods stun or kill their prey with toxic saliva and then tear the prey apart with their strong beak and buccal radula. In fact they resemble modern teleostean fishes to an extraordinary extent in their morphology, physiology, ecology and behaviour<sup>5</sup>.

### Salivary toxins

Anterior salivary glands are associated with the buccal mass and the posterior salivary glands with the digestive gland. Ghiretti<sup>19</sup> isolated a proteinaceous substance from the posterior salivary glands of the cuttlefish, *Sepia officinalis*, which he termed 'cephalotoxin'. It caused paralyzing and respiratory

distress in crustaceans as well as contraction of the digestive tracts of frog and rabbit. Similar toxin showing strong toxicity on crustaceans, mainly crabs was also obtained from two species of octopus, *Octopus vulgaris* and *O. macropus*<sup>20</sup>. Posterior salivary glands from the Mediterranean species of octopus, *Eledone moschata* and *E. aldrovandi*, contain a substance that causes contraction of smooth muscle and hypotension in mammals<sup>21</sup>. Active principle, first called 'moschatin', was later renamed as 'eledoisin'. Authors also found that subcutaneous injection of eledoisin of 25 – 100 µg/kg into an anaesthetized dog stimulated movement of the digestive tract and promoted secretion of the digestive juice. Songdahl and Shapiro<sup>22</sup> extracted a unique neurotoxin from the salivary gland of *Octopus dolfeini* with strong toxicity on crayfish.

The toxin that is secreted from the posterior salivary gland of the blue-ringed octopus, *Octopus maculosus* (= *Hapalochlaena maculosa*) has been named 'maculotoxin' and is derived from the specific name of the octopus<sup>23</sup>. This toxin was earlier thought to be proteinaceous in nature, in analogy with other stinging and biting toxins, but is now recognized to be a small molecule. The toxin is found to be similar to 'tetrodotoxin' and 'saxitoxin' in pharmacological properties being closer to tetrodotoxin in many respects<sup>23,24</sup>. The authors also suggested that maculotoxin was a substance of low molecular weight and that the dose-death relation for maculotoxin in mice coincided well with that for tetrodotoxin. Later, this maculotoxin was identified as tetrodotoxin<sup>25,26</sup>. Sheumack *et al.*<sup>27</sup> had found the occurrence of a tetrodotoxin-like compound in the eggs of the blue-ringed octopus. The biotoxicology of the blue-ringed octopus venom has been detailed by Bonnet<sup>28</sup> with a view to introducing it as a remedy in the Homeopathic *Materia Medica*. Yamashita *et al.*<sup>29</sup> assayed the blue-ringed octopus tetrodotoxin and found that it was present in all body parts and was not associated exclusively with the posterior salivary gland.

Injections of the blue-ringed octopus salivary gland extract and tetrodotoxin into the blue-ringed octopus had no ill-effect on the animals. Similarly, *in vitro* nerve preparations from the animal were not affected by these materials although the extract and toxin are both extremely potent on bioelectrically excitable preparations from other species<sup>30</sup>.

Ueda *et al.*<sup>31</sup> studied the toxicity of the salivary gland extracts of six species of decapodiform

cephalopods. They found three species of cuttlefish venoms to be toxic only to crabs and three species of squid venoms to be toxic to both mice and crabs. A proteinaceous toxin (named SE-cephalotoxin) was also purified from the salivary gland of *Sepia esculenta* and was shown to be a 100 kDa monomeric glycoprotein with a LD<sub>50</sub> value of 2 µg/kg in crab. A full length cDNA coding for SE-cephalotoxin showed it to be a novel proteinaceous toxin<sup>31</sup>.

#### *Body and Liver oil*

Lipids of the short finned squid, *Illex illecebrosus* flesh mainly consist of phospholipids—lecithin and cephalin<sup>32</sup>. The squid, *L. vulgaris* had a lipid load of 25g Kg<sup>-1</sup>, of which approximately 75% were phospholipids<sup>33</sup>. Major saturated fatty acid in both phospholipids and non-phosphorylated lipids was C 16:0 (25% and 21% of total fatty acids). Major unsaturated fatty acid in both lipid fractions was C22: 6n-3 (34% and 23%) followed by C 20: 5n-3 (14% each) in *L. vulgaris*<sup>33</sup>. Oil extracted from the viscera of the cuttlefish (*Sepiella maindroni*) was biochemically analysed for volatile compounds<sup>34</sup>. Composition of fatty acids was monounsaturated fatty acids (50%), polyunsaturated fatty acids (31%) and saturated fatty acids (19%). The total cholesterol was 1.39 mg/100 g oil. The authors considered Hexanal, (E, E)-2, 4-heptadienal, 2-nonanone, benzothiazole, 2-methyl-4-propyl thiazole, 2, 3-butanediol, 1-penten-3-ol and ethyl oleate as principal contributors to the distinctive odour of cuttlefish oil<sup>34</sup>.

Among the invertebrates, cephalopods possess a well defined liver, which constitutes 7-12% of the body weight and has a high oil content ranging from 6 – 40%<sup>35,36</sup>. The fatty acid composition of the squid liver oil is very similar to that of the cod liver oil. The predominant fatty acid in *Illex illecebrosus* is docosahexaenoic acid<sup>37</sup>. Squid and cuttlefish liver is a rich source of n3 polyunsaturated fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)<sup>38</sup>. Joseph<sup>39</sup> reported that cuttlefish (*S. pharaonis*) liver oil contained 38.3% saturated fatty acids, 17.4% polyunsaturated fatty acids and 15.6% monounsaturated fatty acids. Among the polyunsaturated fatty acids, ω-3 PUFAs were 7.6% (EPA-4.9% and DHA – 2.5%), and ω-6 PUFAs were 1.5%.

Diet containing hydrogenated fish oil or hydrogenated beef tallow as the sole energy source induced essential fatty acid (EFA) deficiency in

farmed common carp and rainbow trout. But the replacement of 4-6% hydrogenated oil by cuttlefish or pollock liver oil resulted in the best weight gain and feed conversion<sup>40</sup>. These marine lipids provided necessary levels of EFA. Shyla *et al.*<sup>41</sup> reported that cuttlefish liver oil from *S. pharaonis* can be successfully used as a substitute for conventional lipid sources in the rearing of the giant freshwater prawn, *Macrobrachium rosenbergii*.

Cardio-protective effects of cuttlefish (*S. pharaonis*) liver oil in isoproterenol administered rats was studied by Sherief *et al.*<sup>42</sup>. Authors found that animals fed with 1% liver oil had less incidence of induced heart attack due to the presence of EPA and DHA. Feeding a low dose of cuttlefish (*S. pharaonis*) liver oil can stimulate the immune functions; inhibit inflammatory response and platelet aggregation in rats<sup>43</sup>. Joseph *et al.*<sup>44</sup> studied the antiatherogenic activity of *S. pharaonis* liver oil on cholesterol fed rats. Liver oil significantly reduced total cholesterol, triglycerides, phospholipids, LDL cholesterol and increased the HDL cholesterol in the serum. Supplementation of liver oil with vitamin E and C or green tea flavonoids further enhanced the activity, the most effective combination being liver oil plus flavonoid supplement. The antiatherogenic action was through decreased lipogenesis, increased cholesterol transport to liver, enhanced excretion of neutral sterols and bile acids and, above all, a stimulated antioxidant defense system. Like other PUFAs those present in fish oil can also be easily oxidized, in the absence of antioxidants, to form hydro peroxides and would increase oxidative stress. But unlike  $\omega$ -6 PUFAs,  $\omega$ -3 PUFAs are inhibitors of free radical generation. In the presence of antioxidants like flavonoids, the  $\omega$ -3 PUFAs in cuttlefish liver oil were available to inhibit free radical generation and to thus improve the antioxidant defense system in rats<sup>44</sup>.

### Reproductive strategies

Rocha *et al.*<sup>45</sup> reviewed the reproductive strategies in cephalopods. Cephalopods have highly evolved female and male reproductive systems with elaborate reproductive behaviour like mating and courtship rituals. Fertilized eggs are encased in multiple coatings by the accessory reproductive glands like the oviducal, nidamental and accessory nidamental glands and in some cuttlefish eggs even have an ink coating. Cuttlefish females attach the egg capsules to a common egg mass built up by several females in

shallow coastal waters. Squid eggs are embedded in a common matrix of glandular exudates. Majority of the squids and cuttlefish deposit their eggs and leave them to fend for themselves. It is interesting why these eggs do not fall victim to algal, fungal or bacterial infections. Eggs of cuttlefish and squids appear to resist such infections<sup>46</sup>. Octopuses lay their eggs in a den, which are cared for by the mother or carried by females in their arms, or are incubated in the oviducts<sup>45</sup>.

### Ovarian peptides:

One of the factors responsible for the storage of full grown oocytes in the oviduct before mating and egg release in *Sepia officinalis* has been identified as 5-hydroxytryptamine synthesized in the egg follicles<sup>47</sup>. The first mollusc sperm-attracting peptide was described in *S. officinalis* by Zatylny *et al.*<sup>48</sup>. Benoit Bernay and his co-researchers at the University of Caen and sister Institutes in France investigated the role of ovarian peptides in the cuttlefish, *S. officinalis*. They identified the sepia capsule releasing peptide (Sep CRP-1), a myotropic ovarian peptide released by the fully grown oocyte<sup>49</sup>. Bernay *et al.*<sup>50</sup> described seven related peptides called R-Sep CRPs. This new ovarian peptide family would be responsible for the storage of fully grown oocytes in the genital coelome before mating and play a role in the mechanical secretions of egg capsule products from the main nidamental gland. Bernay *et al.*<sup>51</sup> further investigated three water-borne ovarian peptides in *S. officinalis* whose jelly forming property when resuspended in water could play an important role in the kinetics of peptide diffusion in the external medium. These regulatory peptides were named 'Ovarian Jelly Peptides'. Buresch *et al.*<sup>52</sup> reported the influence of ovary and oviducal gland extracts on male agonistic behaviour in *Loligo pealei*.

### Nidamental gland:

The female reproductive system of certain cephalopod taxa possesses the nidamental gland complex which consists of the paired nidamental glands and the accessory nidamental glands (Fig. 2). The cephalopod nidamental glands are known to play a role in the production of eggs. Pelagic-spawned eggs of the ommastrephid squids such as *Illex* species and *Todarodes* species are wrapped in fragile gels to form the egg masses with a length of 50 cm or more<sup>53,54</sup>. These gels are secreted by the nidamental

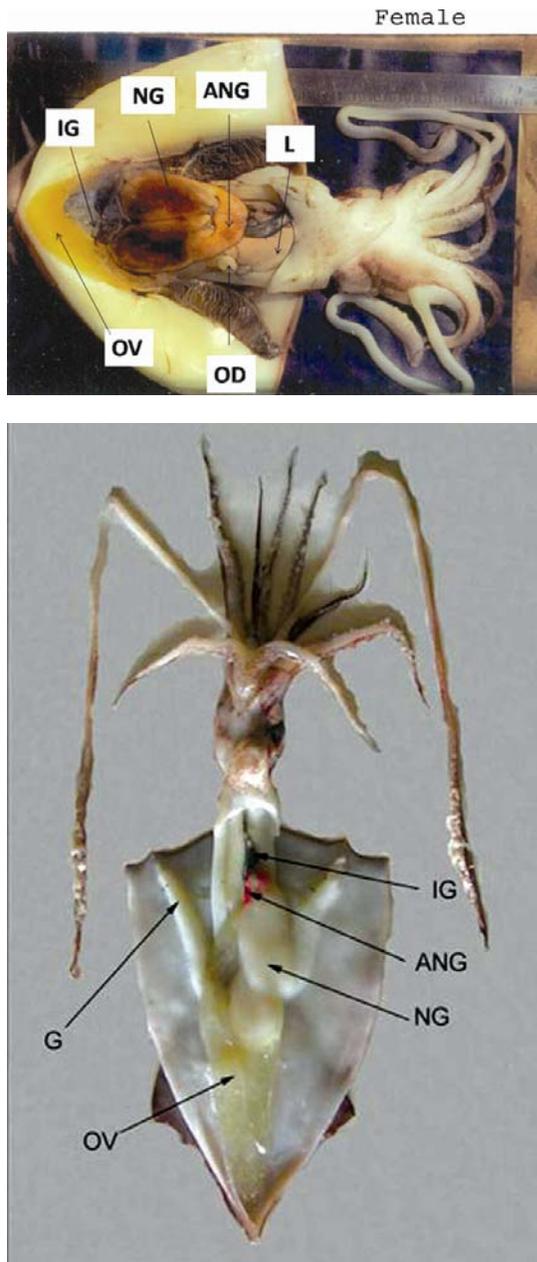


Fig. 2—Cephalopod internal organs  
 (a) Cuttlefish viscera (source: Sherief *et al.*<sup>131</sup>) (ANG- Accessory nidamental gland; IG- Ink gland; L- liver; NG- Nidamental gland; OD- Oviducal Gland; OV- Ovary)  
 (b) Squid viscera (source: Nair J R- personal collection) (ANG- Accessory nidamental gland; G- gill; IG- Ink gland; L- liver; NG- Nidamental gland)

glands and serve as a physical barrier between the eggs and the surrounding water. Further, the exudate appears to trigger sperm release from implanted spermatophores<sup>54</sup> and also function as a buoyant mechanism<sup>55</sup> in *I. illicebrosus*. Spawned eggs of the squid *I. argentinus* are assumed to be wrapped up in

fragile gels, which are largely derived from the mucosubstance of nidamental glands<sup>56</sup>. Once secreted, the mucosubstance forms a highly hydrated gel comprising a large number of constituents to offer more perfect protection for the eggs. Sugiura and Kimura<sup>57</sup> found the salt-soluble component of mucosubstance to be a mucin complex, which presumably existed in the gel structure of egg mass in *I. argentinus*.

Atkinson and Granholm<sup>58</sup> and Atkinson<sup>59</sup> demonstrated an anticiliary activity due to an immobilising factor in the nidamental gland secretions and also in the capsule sheath extract of *Loligo pealei* eggs. This factor agglutinates the cilia of motile metazoans and may inhibit the protozoan predators of the eggs. Sheath bacteria from the accessory nidamental gland, in conjunction with the ‘immobilising factor’ of the nidamental gland might constitute part of an interesting system of embryonic defense in squid and cuttlefish.

#### Accessory nidamental gland (ANG)

The ANGs are associated with egg laying and they harbour dense bacterial communities<sup>60</sup>. It was reported that the gland in *Loligo pealei* has many of the structural features of a secretory organ and the presence of three types of tubules - red, white and yellow, with the tubules of similar colour clustered together. This gave the gland a mottled appearance. In *L. opalascens*, the ANGs displayed tubules composed of a single layer of epithelial cells and expressed numerous cilia and microvilli highlighting the secretory nature of the gland<sup>61</sup>. The electron microscopic studies on the ANG of the cuttlefish *S. pharaonis* revealed the presence of numerous globules at the secretory sites on the luminal surface of the tubules<sup>62</sup> (Fig. 3).

#### Symbiotic Bacteria:

Microbial symbioses are essential for the normal development and growth of animals. A phylogenetically diverse assemblage of bacteria is harboured in the ANG of loliginids<sup>63-65</sup>. Bacterial associates of cephalopods were previously described in terms of morphology<sup>60,66</sup> and pigmentation<sup>67,68,66</sup>. The symbiotic ANG-bacteria have been identified in recent studies by bacterial 16S RNA gene (rRNA) sequencing<sup>64,69-71</sup>. Symbiotic bacterial community of loliginid squids was mainly constituted by  $\alpha$ -proteobacteria (*Roseobacter*, *Agrobacterium*,

*Silicibacter*, *Stappia*, *Rhodobium*-like), of  $\gamma$ -proteobacteria (*Vibrio*, *Shewanella*, *Pseudoalteromonas*) and of *Cytophaga-Flavobacteria-Bacteroides*-like strains<sup>71</sup>. The parallel analysis of cephalopod phylogeny and ANG's symbiotic bacterial strains suggests high specificity of gram positive strains to cephalopod taxonomy at higher taxonomic levels. They may represent interesting candidates for co-evolutionary studies<sup>71</sup>.

Exogenous and endogenous factors controlling sexual maturation in cephalopods was reviewed by Mangold<sup>72</sup>. Colour of the ANG was one of the major characters for quantifying animal maturity stages in *L. pealei*<sup>73,74</sup>; in *L. vulgaris*<sup>75</sup>; in *L. forbesi*<sup>76,77</sup>; and in *L. duvauceli*<sup>78</sup>. The red colour of the ANG was a clear indicator of the sexual maturity of the female cuttlefish, *S. officinalis*<sup>79</sup>. Bacterial community

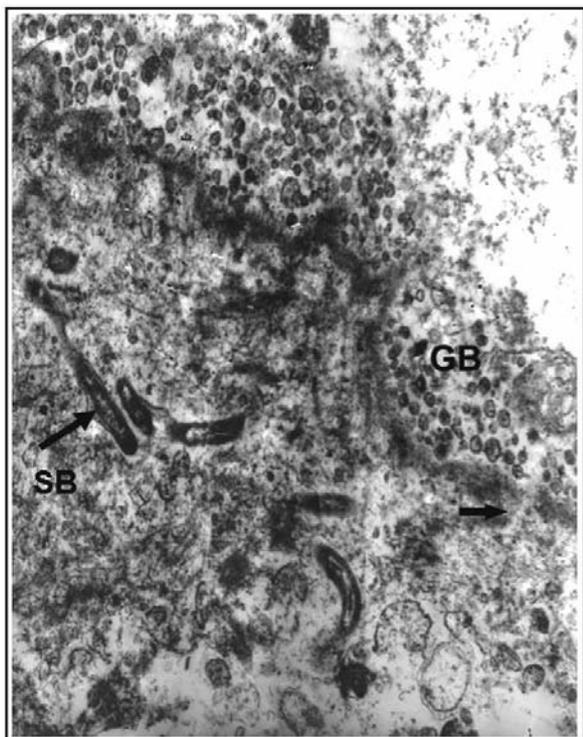


Fig. 3—EM section of ANG of *Sepia pharaonis* (x 25,000) (GB- Globular bodies; SB- Symbiotic bacterium) (source: Nair *et al.*<sup>62</sup>)

accumulates carotenoid pigments during sexual maturation of *S. officinalis*<sup>68</sup> and of *L. pealei*<sup>63</sup>. Declair and Richard<sup>80</sup> had named the *S. officinalis* ANG novel carotenoid as 'sepiaxanthine'. Authors found the pigments tightly bound to granules which are actively secreted by the gland. Gomathi<sup>78</sup> studied the histology of the ANGs in the different stages of animal maturity in the Indian squid, *L. duvauceli*. She found that the process of accumulation and secretion of the pigment bound granules from the ANG closely followed the ovarian maturation cycle in the Indian squid.

Symbiotic bacteria (*Alteromonas* strain) in *L. pealei* were orange-red pigmented<sup>64</sup>. *Rhodobium-Xanthobacter* and *Roseobacter* strains are phototropic and could be responsible for the red-orange colour of the ANG in the mature *S. officinalis* females due to the accumulation of carotenoids<sup>70</sup>. Absorbance spectrum of the ripe ANG extracts of different species of squids is given in Table 1.

Values within the brackets indicate the respective absorbance.

Pierantoni<sup>81</sup>, way back in 1918, deduced a possible 'vertical transmission' of ANG bacteria from generation to generation. But Lum-Kong and Hastings<sup>63</sup> and Kaufman *et al.*<sup>61</sup>, working with *L. forbesi* and *L. opalascens*, suggested 'horizontal transmission' of the bacteria from the environment. The absence of bacteria (aposymbiotic) in the embryos of *Sepioteuthis lessoniana* and *L. vulgaris* would support this hypothesis<sup>71</sup>.

However, though loliginid embryos lack bacteria, the egg capsules harbour dense populations of bacteria, most of which were also present in the ANG<sup>61,69,71</sup>. Studies of Biggs and Epel<sup>82</sup> working on *L. opalascens* suggested that ANG contract during the formation of the egg capsule, periodically expressing its bacterial contents into the sheath. A possible role of egg capsule sheath bacteria was the defense of the developing embryos. Sheath bacteria might populate the layers of the capsule sheath so heavily that available resources are exhausted to the detriment of pathogenic organisms. It is possible that

Table 1—Absorbance spectrum of ripe ANG extracts of squids

Sl. No	Species	$\lambda_{max}$		$\lambda_{max}$		Authors
1	<i>Loligo pealei</i>	—	—	485.0-490.0	510.0-520.0	Bloodgood <sup>60</sup>
2	<i>L. forbesi</i>	—	335.0	496.0	526.0	Lum-kong & Hastings <sup>63</sup>
3	<i>L. duvauceli</i>	—	334.0(0.0748A)	494.5 (2.123A)	525.5 (1.665A)	Sherief <i>et al.</i> <sup>81</sup>
4	<i>L. duvauceli</i>	290.5 (1.314A)	315.5 (0.985A)	498.5 (2.597A)	528.0 (2.111A)	Gomathi <sup>78</sup>

the bacteria do not 'passively compete', but also actively produce an antimicrobial compound<sup>82</sup>.

#### *Antimicrobial activity:*

Benkendorff *et al.*<sup>83</sup> suggested that the egg masses of marine molluscs appear to have broad spectrum antimicrobial activity with gram positive bacteria showing comparatively more susceptibility. A preliminary experiment by Barbieri *et al.*<sup>65</sup> has shown that butanol-ANG extract from *L. pealei* could inhibit the growth of marine bacterial pathogens like *Vibrio anguillarum* and *Streptomyces griseus*. They attributed this antibacterial function to the ANG-symbionts, *Alteromonas* and *Shewanella*. These bacterial strains were not detected in the ANG of *S. officinalis* but according to Grigioni *et al.*<sup>70</sup>, antimicrobial activity by other symbiotic bacterial strains in *Sepia* spp can not be excluded. Sherief *et al.*<sup>84</sup> reported antibacterial activity in the ripe ANG-butanol extracts of *S. pharaonis*, *S. aculeata*, *Sepiella inermis*, and *L. duvauceli* against *E. coli*, *Aeromonas* sp, *S. aureus* and *B. megaterium*. Significantly higher levels of unsaturated fatty acids in the ripe ANG could be the factor responsible for the antibacterial activity in *S. pharaonis*<sup>84</sup> and in *L. duvauceli*<sup>85</sup>. Major unsaturated fatty acids ( DHA, oleic acid, arachidonic acid and EPA) content was 1.973 mg/g tissue in the ripe stage extract, where as in the immature stage extract which did not show antibacterial activity, it was only 0.251 mg/g tissue in *L. duvauceli*<sup>85</sup>. A halide dependent peroxidase occurs abundantly in the ANG of the squid, *Euprymna scolopes*. This enzyme functions not only to control pathogens, but also to modulate interactions of the host animals with their beneficial partners<sup>86</sup>.

#### *Squid-Vibrio association:*

For the past two decades Professor Margaret McFall-Ngai and her co-workers have been extensively investigating the symbiotic relationship between the Hawaiian bobtail squid (*Euprymna scolopes*) light organ and the luminous bacterium, *Vibrio fischeri*. The bacterium gets food and shelter from the squid in exchange for making light. The host establishes and maintains stable beneficial association with bacteria in two tissue types, the light organ and the ANG. In the light organ, the host houses symbiosis competent strains of the luminous bacterium, *Vibrio fischeri*, which grows extracellularly within epithelia lined crypts of the adult light organ<sup>87</sup>. The organ is surrounded by

accessory tissues, including diverticula of the ink sac, a reflector and a muscle-derived lens<sup>87</sup>, that serve to modify the bacterial luminescence for use by the host in its nocturnal antipredatory behaviour<sup>88</sup>. The squid was found ejecting about 90% of the bacterial cargo each morning when they descend to deeper waters for protection. The remaining bacterial load multiplies during the day and by nightfall the squid contains enough bacteria to switch on that protective flashlight as they head up to feed in the surface waters<sup>89</sup>.

The symbionts must be acquired from the environment (horizontal transmission) during each generation. Identification of the relevant symbiotic partner against a myriad of unwanted relationships is a formidable task. The genetic mechanism governing this specificity has been revealed in the squid-*Vibrio* symbiosis by Mandel *et al.*<sup>90</sup>. The authors found that a two component sensor kinase RscS is necessary and sufficient for conferring efficient colonisation of *E. scolopes* squid by bioluminescent *V. fischeri* from the North Pacific Ocean. In the squid symbiont *V. fischeri* ES 114, RscS controls light-organ colonization by inducing the Syp exopolysaccharide, a mediator of biofilm formation during initial infection<sup>90</sup>. During the two decades of research a large number of review articles have been published on the usefulness of this squid-*Vibrio* model in understanding animal-bacterial partnerships<sup>91-96</sup>.

## **Defense mechanisms**

### *Inking:*

Inking by the cephalopods has long been recognized as an adaptive response to predation and physical threat, by means of a combination of mechanisms that include chemical deterrence, sensory disruption and phago-mimicry. Cephalopod inks are chemical secretions produced by and released from the ink sac, which is not a homologue of the ink glands of gastropods but a modified hypobranchial gland<sup>97,98</sup>.

Cuttlefish ink consists of melanin granules in a viscous colourless medium<sup>99</sup>. The melanin pigment is manufactured in the mature cells of the ink gland<sup>100,101</sup>, a highly specialized organ situated at the bottom of the ink sac and deputed to continuous production of the ink. At the end of the maturation process, ink gland cells degenerate and shed their contents into the ink sac, which acts as a reservoir of the exhausted material<sup>99</sup>. Melanin isolated from the ink sac of *S. officinalis* (*Sepia melanin*) has been

proposed as a standard for natural eumelanin<sup>102</sup>. Melanogenesis in the ink sac of *S. officinalis* seems to follow the general scheme of melanin formation in vertebrates<sup>103,104</sup>. Natural sepia ink is a powerful dye made from the ink of the cuttlefish.

The morphology of released ink is of two types: pseudomorphs and clouds. Pseudomorphs are well defined objects composed of ink and mucous. They keep their form and physical integrity for sometime after release and can be almost as large as the individual releasing them<sup>5,105,106</sup>. They are generally thought to function in defense as a visual stimulus. This may be in two ways: as a dispersed 'smoke screen' behind which the cephalopod can escape unseen, especially true of clouds; or it might be as a distracting 'decoy' that attracts the attention of the predator while the animal escapes, especially true for pseudomorphs<sup>5,107,105</sup>. An example of the use of decoy is the 'Blanch-Ink-Jet maneuver' described by Hanlon and Messenger<sup>5</sup>.

Prota *et al.*<sup>107</sup> suggested that the occurrence of large amounts of tyrosinase in the ejected ink of cephalopods would ensure efficient conversion of catechols into toxic quinones acting as a deterrent for the predator. Lucero *et al.*<sup>108</sup> postulated that in the squid, *L. opalascens* the ejected ink functions as a warning signal or alarm substance that confuses predators and alerts conspecifics to the presence of danger. The authors identified two metabolites, L-Dopa and dopamine effector molecules in concentrations sufficient to produce physiological effects. They also suggested that an unidentified antioxidant in the ink may prevent rapid oxidation of the metabolites following dilution in seawater<sup>108</sup>.

The biosynthesis, localization and fate of catecholamines in the ink gland of the cuttlefish *S. officinalis* were investigated by Fiore *et al.*<sup>109</sup>. HPLC analysis of crude ink gland extract indicated the presence of dopa ( $2.18 \pm 0.8$  nmol/ mg of protein) and dopamine ( $0.06 \pm 0.02$  nmol/mg of protein). The dopamine in secreted ink was adsorbed on to melanin granules, preventing excessive dilution after ejection, thus ensuring efficient interactions with target organs in dopamine-mediated inter- and intraspecies communication<sup>109</sup>.

Derby *et al.*<sup>110</sup> working on six species of cephalopods (squids, cuttlefish and octopuses) found millimolar levels of total free amino acids (FAA) and ammonium in the ink gland secretions. The FAAs in highest concentrations were taurine, aspartic acid,

glutamic acid, alanine, and lysine. Crustaceans and fish, which are the major predators of these cephalopods, have specific receptor systems for these FAAs. The authors concluded that the inking mollusks have the potential to use sensory disruption and/or phagomimicry as a chemical defence<sup>110</sup>. Derby<sup>111</sup> has also reviewed the chemical mechanisms of defence in inking mollusks.

#### *Ink bioactives*

Cuttlefish ink has wide applications in homeopathic medicine (medicinal name-sepia). Sepia is one of the major contributions made to Homeopathy Materia Medica by Dr. Hahnemann. The source of this medicine is *S. officinalis*. Sepia is used to treat hormonal imbalances especially in women. It is indicated for all possible gynecological, urinary tract infections and pregnancy related complaints<sup>112</sup>. Cuttlefish ink also has a rich history in ancient Roman and Greek medicine. In Rome it was used for baldness and in Greece as a cure for kidney gravel and gonorrhoea. Cuttlefish ink is a traditional Chinese medicine listed in the Compendium of Materia Medica compiled by Shizhen Li, at the time of the Ming Dynasty and first employed to treat heart pain<sup>113</sup>.

Mimura *et al.*<sup>114</sup> reported that melanin extract obtained from the squid ink could inhibit gastric secretion in rats. The extract mainly contained a melanoprotein composed of melanin pigment (90%), protein (5.8%) and carbohydrate (0.8%). The authors<sup>115</sup> further found that melanin obtained from the ink bags of *Octopus vulgaris* inhibited gastric secretion in rats and prevented ulcer formation in pylorus ligated rats.

In Japan the squid ink has traditional application in food products<sup>116</sup>. The cuttlefish ink is believed to exhibit antiseptic effect on 'ika-shiokara', a cured cuttlefish meat product. It has been shown that product treated with the ink had an extended shelf life<sup>117</sup>. Food chemistry of the ink of the neon flying squid, boreal clubhook and boreopacific gonate squid revealed that the squid inks were rich in taurine and hydroxyproline<sup>118</sup>.

Purified extract of the cuttlefish, *Sepioteuthis lessoniana* is reported to have antibacterial activity against *Staphylococcus aureus*<sup>119</sup>. Similar findings against gram negative bacteria were made by Nirmale *et al.*<sup>120</sup> using freeze dried ink of *L. duvauceli*. Chacko and Patterson<sup>121</sup> described the antibacterial activity of

*S. pharaonis* ink extract. Aqueous extracts from the cephalopod ink were tested against Molony Murine Leukemia Virus Reverse Transcriptase (MMLVRT) and have exhibited antiretroviral activity<sup>122</sup>. Ink from the juveniles of *S. inermis* and *L. duvauceli* showed strong inhibition of MMLVRT.

Takaya *et al.*<sup>123</sup> investigated the antitumour activity of a peptidoglycan fraction from the squid (*I. argentinus*) ink against Meth A fibrosarcoma in mice. The fraction contained 7.8% peptide, 57% polysaccharide and 30% pigment. The authors<sup>124</sup> described novel fucose rich glycosaminoglycans from squid ink bearing repeating unit of trisaccharide structure (-6 GalNAc-3GlcAB1-Fucal-)n. Lu *et al.*<sup>125</sup> working with cuttlefish ink and mice found increased humoral immunity in ink treated mice. The antitumour fraction of the *I. argentinus* ink was separated by Phenyl Sepharose CL-4B chromatography into three fractions: illexin peptidoglycan, tyrosinase, and the complex of the two<sup>126</sup>. The third fraction containing the illexin peptidoglycan and tyrosinase showed the highest activity against Meth A tumour in BALB/c mice, suggesting the role of both components in antitumour activity of squid ink. Naraoka *et al.*<sup>127</sup> did further purification, characterization and molecular cloning of squid tyrosinase from *I. argentinus*. The melanin free ink of the cuttlefish, *S. officinalis* is shown to have toxic effect on a variety of cell lines and the active factor was identified as tyrosinase<sup>99</sup>. Purified *Sepia* tyrosinase was found to induce a significant increase in caspase 3 activity in PC 12 cells, leading eventually to an irreversible apoptotic process. The results disclose a hitherto unrecognized property of tyrosinase that may lead to a reappraisal of its biological significance beyond that of a mere pigment producing enzyme<sup>99</sup>.

Crude ink of the cuttlefish, *S. pharaonis* was evaluated for its toxicity on chick embryo<sup>128</sup>. The ink showed significant inhibitory effect on the development of the embryo and induced DNA fragmentation in treated embryos. The antitumour activity of the different peptidoglycan fractions of *S. pharaonis* ink in Dalton's Lymphoma Ascites (DLA) bearing mice showed increased activity with increasing purification<sup>129</sup>. The most potent fraction was found to be an uronic acid rich polysaccharide forming 85% of the peptidoglycan. The purified peptidoglycan fraction was also found to inhibit the growth of human cervical cancer (HeLa and Caski)

cell lines<sup>130,131</sup>. It is suggested that the antiproliferative effects of the purified fraction were mediated through apoptosis. The purified peptidoglycan induced typical morphological characters of apoptosis like loss of membrane integrity, chromatin condensation, membrane blebbing and DNA damage in the cancer cells.

The protective and therapeutic effects of cuttlefish ink on hemopoiesis in <sup>60</sup>Co  $\gamma$  radiated model female ICR mice were investigated by Lei *et al.*<sup>132</sup>. The ink could promote the proliferation and the differentiation of granulocyte-monocyte progenitor cells and enhance non-specific immunity and specific immunity significantly. The mechanism may be that cuttlefish ink weakens the irradiation injury on hemopoietic microenvironment and cells via regulating immunological function, inducing Gm-CSF and other cytokines and elevating SOD activity in mice<sup>132</sup>. Zhong *et al.*<sup>113</sup> found that the cuttlefish (*S. officinalis*) ink extract can reverse the spleen damage and marrow hemopoiesis induced by cyclophosphamide and thus protect the body from chemotherapeutic injury in studies on BALB/c mice.

#### Camouflage colouration

Cephalopods are experts in the art of camouflage (masters of disguise) with a highly evolved neurally controlled camouflaging mechanism of their skin. Each layer of the cephalopod skin has a specific function. The outermost layer is full of chromatophores (red erythrophores; yellow xanthophores and black/brown melanophores). Innermost layer uses light-scattering leucophore cells to reflect ambient light. Between these layers is the reflective layer of skin made up of iridophores. These cells reflect colour and are responsible for the blue, green, golden and silver shades in the animal's skin. They are under the control of the 'muscarinic cholinergic system' and the associated neurotransmitter 'acetylcholine' acting as a hormone in *Lollinguncula brevis*<sup>133</sup>. Mathger *et al.*<sup>134</sup> reviewed the recent anatomical and experimental evidence regarding the mechanisms of reflection and diffusion of light by the different cell types (iridophores and leucophores) of various cephalopod species. The authors illustrated how structural colouration contributes to the overall appearance of the cephalopods during intra- and interspecific behavioural interactions including camouflage. Mathger *et al.*<sup>135</sup> reported that the skin of the cuttlefish, *Sepia officinalis* contains opsin transcripts

suggesting a possible role of distributed light sensing by skin, in addition to visual sensing. The skin opsin may provide an explanation for how cuttlefish can achieve their impressive camouflage and signaling body patterns<sup>135</sup> in the absence of colour perception<sup>136</sup>.

#### *Reflectin-protein:*

The reflectance arises from proteins known as 'reflectins'. A family of unusual proteins is deposited in flat, structural platelets in reflective tissues of the squid *Euprymna scolopes*<sup>137</sup>. The reflectin-protein based platelets are assembled into lamellar thin-film reflectors called 'iridosomes' contained within iridescent cells called 'iridocytes'. These proteins are encoded by at least six genes in three subfamilies and have no reported homologs outside of squids. The proteins have a very unusual composition, with four relatively rare residues (tyrosine, methionine, arginine, and tryptophan) comprising approximately 57% of a reflectin, and several common residues (alanine, isoleucine, leucine, and lysine) occurring in none of the family members<sup>137</sup>. These protein-based reflectors in squids provide a marked example of nanofabrication in animal systems. Kramer *et al.*<sup>138</sup> investigated the property-function relationships of the unique family of reflective proteins. They demonstrated that reflectin can be easily processed into thin films, photonic grating structures and fibres. Izumi *et al.*<sup>139</sup> sequenced and characterized three new members of the reflectin family associated with iridescence in squid. They concluded that tyrosine phosphorylation of reflectin proteins is involved in the regulation of dynamic iridescence in *Loligo*. Tao *et al.*<sup>140</sup> cloned and expressed a specific reflectin protein found in the responsive iridophore cells of the squid, *L. pealei*, which are unique in their ability to switch on/off and change colour. They demonstrated that these iridophores can be chemically tuned to reflect the entire visible spectrum. This is facilitated by the hierarchical assembly of nanoscale protein particles that elicit large volume changes upon condensation<sup>140</sup>. These findings provide insight into the design and synthesis of biomaterials for complex responsive functions in optical applications.

#### **Conclusion**

Since the 1980s laboratory culture and later on mariculture of cephalopods for use as experimental animals for biomedical research has been active<sup>10,141,142</sup>. In this context an interesting research

publication that caught our attention was that of Moltchaniwyskyj *et al.*<sup>143</sup> which collates recent literature that provides details of collection methods, handling, maintenance and culture of a range of cephalopods and their use as experimental animals. The paper discusses the ethical and welfare considerations when using cephalopods as experimental animals. They cite a number of factors, including morality, quality of information derived from experiments and public perceptions that should drive the motivation to consider welfare issues. The authors of the publication hail from laboratories in Australia, South Africa, Brazil, USA, Japan, Spain and Germany. They conclude that refinement of methods and techniques should be a major step in ensuring protection of cephalopod welfare in both laboratory and field studies. We feel that this aspect merits greater scientific debate because of the biological and behavioural complexities exhibited by these highly evolved, lovable invertebrates. A case in point is 'Paul' the octopus (*O. vulgaris*) of the Sea Life Centre in Oberhausen, Germany whose antics caught the peoples attention the world over.

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