An olfactory facility for the detection and processing of molecular signals in their environment is present in both vertebrate and invertebrate animals. In all animals, such systems follow a remarkably similar anatomical and functional plan in which chemical signals bind to receptor sites on the membranes of olfactory receptor neurones that, in turn, project exclusively to brain areas with glomerular-structured neuropile (see reviews by Ache, 1991; Hildebrand, 1995). Crustacean olfactory systems conform to this overall plan. Their olfactory receptor neurones (ORNs) are located in clusters of approximately 100 cells beneath unique hair-like sensilla called aesthetascs, found only on the distal segments of the outer flagella of the antennules (Tierney et al. 1984, 1986).

A significant body of information exists on the ORNs, on their central projections and on the anatomy and physiology of some central neurones in the adults of several decapods (Schmidt and Ache, 1992; Mellon and Alones, 1993, Sandeman and Sandeman, 1994; Wachowiak and Ache, 1994; Sandeman et al. 1995). Much less is known about the time of appearance of ORNs and aesthetasc sensilla in the antennules of crayfish embryos. We have followed the embryonic and postembryonic development and the addition of aesthetasc hairs during the animal’s lifetime by comparing freshly moulted exuvia with their newly emerged antennules. We conclude that, in both juveniles and adults, ORNs and aesthetasc sensilla are added at the proximal end of the receptor array, about halfway along the flagellum.

Key words: development, crustacean, olfaction, receptors, *Cherax destructor*.
the ORNs and aesthetasc sensilla is visible in unfixed and unstained whole mounts using differential contrast microscopy. The development of the ORNs was followed in individual pre- and post-moult animals in this study. Silver impregnation (Blest and Davie, 1980) of sectioned embryonic and postembryonic antennules aided the identification of developing ORNs.

The comparison between the exuvia and newly moulted antennules provided some of the most useful information for this study. The intermoult period of the postembryonic stages POI and POII is well known (Sandeman and Sandeman, 1991) so that they could be examined very shortly before they moulted. In this condition, the exuvia has often already parted from the antennule and a direct comparison can be made between the segments and sensilla of the antennule and those of the exuvia. Moult ing in the older animals often occurs during the daylight hours, and we were able to retrieve the exuvia before the newly moulted animal consumed it. The antennules of such animals were removed and fixed with their matching exuvia in 70% ethanol. Antennules, matching exuvia and all sensilla were then drawn using a stereomicroscope camera lucida and compared with one another, segment by segment. A minimum of 10 animals from each stage was examined. Scanning electron microscope (SEM) photographs were used to confirm the results obtained from the light microscope. Material for the SEM was fixed in 70% alcohol, dehydrated in a graded alcohol series, cleared in xylene and placed in a dust-free container to allow the xylene to evaporate. Dry antennules were mounted on stubs covered with double-sided black carbon tape (Alltech adhesive carbon tape G 3939), sputter-coated with gold and viewed with a Leica Cambridge SEM at 20 kV.

The embryonic development of Cherax destructor has been staged as percentages using anatomical criteria (Sandeman and Sandeman, 1991). Egg deposition is defined as 0% and hatching as 100% development. At 20°C, this takes 40 days. The hatching is the first postembryonic stage (POI) and is attached to the mother’s swimmerets for 5–7 days until the next moult. The second postembryonic stage (POII) clings to the swimmerets of the mother for another 14 days before moult ing again to produce the first adult stage, ADI. ADI animals move about over the mother’s body and will leave her for short periods. Approximately 14 days later, the ADIs moult into the second and independent adult stage ADII. From then on, animals grow in size by alternating moult and intermoult periods throughout their lives.

Results

The first antennae, or antennules, in freshwater crayfish each consist of the coxopodite, basipodite and ischiopodite which bears two flagella, one lateral and one medial. The three basal joints contain muscles, and those in the ischiopodite move the flagella. The flagella themselves consist of a large number of annular segments connected by arthrodial membranes and are free of muscle tissue. In the adults, both flagella bear mechanoreceptive hairs, most of which fall into three categories: feathered hairs that lie flat against the shaft of the antennule; and small and large smooth-shafted guard hairs that project at right angles to the long axis of the antennule. At higher magnification, the shafts of the guard hairs can be seen to be sculpted into many small protuberances. The large guard hairs are segmented (Fig. 1A,B,C). The distal segments of the lateral flagellum bear the characteristic thin-walled chemoreceptive aesthetasc sensilla. The aesthetasc sensilla occur in transverse rows containing between two and five sensilla. No segment of the Cherax destructor flagellum ever has more than a single row of sensilla, in contrast to the situation in Orconectes propinquus or Procambarus clarkii which have two rows of sensilla per segment (Tierney et al. 1986; Mellon and Alones, 1993). Dendrites of the ORNs extend into the shafts of the aesthetascs, which have a thin cuticular wall permeable to dyes (Tierney et al. 1986) and to radioactive leucine (Sandeman and Denburg, 1976; Mellon et al. 1989).

Development of the olfactory receptor neurones and aesthetasc sensilla

The antennules, second antennae and small mandibles can be identified as single lobe-like appendages in the head region of embryos that have reached 40–50% development. The antennules are unsegmented and are nearly equal in length to the unsegmented second antennae (Fig. 2A). The first indication of the biramous adult form of the antennules appears at 55% development with small protuberances visible about halfway along the medio-caudal edges of the antennule, indicating that the lateral antennular flagellum is the first to differentiate. There is no segmentation at this stage (Fig. 2B).

At 60% development, the lateral flagellum of the antennule has lengthened to keep pace with the second antenna, but the bud of the medial flagellum remains relatively small. Oval aggregations of cells at the most distal end of the lateral flagellum signal the beginning of the first olfactory receptor neurone clusters and confirm that differentiation of the antennule begins at the distal end of the lateral flagellum. The first signs of segmentation at the base of the antennule appear as small indentations proximal to the lateral and medial flagellar bifurcation.

By 85% development, antennular development has proceeded to the point where five clusters of ORNs can be identified in the lateral flagellum, the most distal being the oldest and most advanced, the others being added proximally (Fig. 2C). The three basal joints are now more clearly indicated by indentations of the cuticle. The medial flagellum is approximately half the length of the lateral flagellum and their proximal bases rest on the most distal of the common segments, the ischiopodite.

At hatching, the POI crayfish is virtually bare of external receptor hairs (Fig. 3A). The antennules are clearly differentiated into three basal segments and two flagella, the lateral still larger than the medial. The lateral flagellum has five segments; a distal segment and four shorter more proximal segments (Fig. 3B).
Clusters of ORNs lie beneath the transparent cuticle of the lateral flagella. The distal segment of each flagellum contains three such clusters. Given that differentiation of the flagellum proceeds from the distal end and that the distal segment is the oldest, we refer to this as the first flagellar segment and number the flagellar segments in ascending order from distal to proximal.

Aesthetasc sensilla materialize beneath the cuticle between day 0 and day 5 of the POI stage and can be clearly discerned in the three most distal segments, just prior to moulting (Fig. 4A). The distal segment contains three aesthetascs, the next proximal segment one aesthetasc and the third segment from the tip, one or two aesthetascs. Outlines of the aesthetascs in the most proximal segments of the lateral flagellum are visible but appear not to be as advanced as in the more distal segments. The segments of the lateral flagellum lengthen beneath the cuticle during the 5–7 day intermoult period of the POI animals. Three new segments (6, 7 and 8) appear immediately distal to the ischiopodite and proximal to the five existing segments.

On day 6 at 20 °C, the POI moults to emerge as a POII animal with external receptor hairs and aesthetasc sensilla on the antennules. The lateral flagellum of the POII stage contains 7–8 segments. Heralded by their subcuticular appearance in the POI stage, segment 1 of the POII lateral flagellum bears three
external aesthetasc sensilla. Segment 2 carries one sensillum, and segment 3 has one or two sensilla (Fig. 4B). Aesthetasc on segments 4 and 5 are clearly established beneath the transparent cuticle and less clearly defined in segments 6, 7 and 8.

The POII moults into the ADI after 14 days and the aesthetasc sensilla on segments 4 and 5 emerge as free sensilla. Another two segments are added immediately distal to the ischiopodite, the aesthetasc sensilla beneath the cuticle of segments 6, 7 and 8 mature, and the ADI crayfish typically possesses a lateral antennular flagellum with 8–9 segments. The ADI animals show the typical ‘flicking’ behaviour of the antennules and, for the first time, search for food and eat it. Five receptor hairs, possibly mechanoreceptors, emerge at the very tip of segment 1 of the lateral flagellum. Similar receptor hairs appear lateral to the aesthetasc on segments 2, 3 and 4.

The development of the aesthetasc hairs was followed over the next three stages, i.e. ADII, ADIII and ADIV, and was seen to conform to the above plan, new segments always appearing at the base of the flagellum (Fig. 5). The intermoult period increases beyond the ADIV stage to a point at which it becomes impractical to follow individual animals.

Nevertheless, counts of the aesthetasc sensilla in animals with carapace lengths ranging from 7 to 40 mm show that the
number of segments carrying aesthetasc sensilla increases continually, as does the average number of aesthetasc on each segment (Table 1).

The numbers of aesthetasc per segment also varies in relation to the position of the segment along the flagellum. In ADI to ADIV crayfish, the distal segments are characterised by the 3, 1, 2, or 3, 1, 1 pattern carried over from the postembryonic stages. As the animals grow, this is replaced by a pattern in which the distal segments generally carry fewer sensilla than the more proximal segments. In all cases, however, the most proximal aesthetasc-bearing segment has fewer sensilla than those immediately distal to it. The gradual increase in the numbers of aesthetasc per segment during growth of the animal could be the result of the lateral addition of the aesthetasc to the segments at each moult or of the shedding of the older more distal segments and the replacement of these at the proximal end by segments bearing more sensilla, or both. The loss of the 3, 1, 2 pattern of the juveniles at an early stage would support the idea of distal shedding, as would the presence in the larger animals of a greater number of aesthetasc on the distal segments.

Growth and turnover in the adult

The distribution of the guard and feather hairs along the lateral flagellum of the antennule in Cherax destructor is of considerable importance for this study because it provides a way to identify individually each segment along the flagellum. At the proximal end of the flagellum, very few guard hairs are present and the feathered hairs surround each annulus. More distally, and several segments before the proximal end of the aesthetasc array, we find a greater proportion of guard hairs and the absence of the feathered hairs on the ventral surface of the segments. Some segments here have one or two aesthetasc sensilla and small swellings across the segment in line with any aesthetasc present.

The presence of the small or large guard hairs on segments bearing aesthetasc sensilla is variable. Some aesthetasc-bearing segments have no guard hairs, some have only small or only large guard hairs and some have both. SEM photographs show that segments without guard hairs do not have scars from the possible loss of such hairs. The particular complement of guard hairs on any segment does not follow any repeated or predictable pattern along the length of the flagellum so that, as a sequence, each flagellum is unique.

Three possibilities follow from this observation in relation to the addition of ORN aesthetasc sensilla at moulting in adults. (1) If ORNs and aesthetasc sensilla are added only at the proximal end of the receptor array, as in the juveniles, then particular segments of the distal ends of the exuvium and flagellum will match if they are placed side by side. In addition, if some distal segments are shed at the distal end, the exuvium should protrude beyond the new flagellum when the two are aligned at their matching point. (2) If ORNs and aesthetasc are added distally, the proximal segments of exuvium and flagellum will match and, at the matchpoint, the new flagellum should protrude beyond the exuvium. (3) If new aesthetasc and guard hairs are added along the entire receptor array, it will not be possible to match the segments of the exuvium and the new flagellum.

The comparisons were carried out by first recording, in horizontal columns, the numbers of receptors on aesthetasc-bearing segments of both exuvia and flagellum. The columns were then placed alongside one another with the most proximal aesthetasc-bearing ‘segments’ in register (Fig. 6). ‘Segments’ of the exuvium that carried the same number of a particular receptor type (i.e. small guard hairs, large guard hairs or aesthetasc) as their corresponding flagellar segments were tallied and expressed as a percentage of all the segments compared. The exuvial column was then shifted by one segment past the flagellar column and all perfectly matching segments were tallied as before. The exuvial column was first moved ‘distally’ past the antennule and then ‘proximally’ from the starting point.

Exuvia of 10 animals ranging from 35 to 55 mm in carapace length were compared with their newly moulted flagella. As a control, flagella from the left side were compared with those from the right side on the same animals.

The comparison using the numbers of large guard hairs produced the clearest result. Here, the possibilities are limited to the presence of no hairs, one hair or two hairs. To test the method, two columns of numbers were chosen at random between 0 and 2 and compared as described above.
Predictably, we found an average match of 33% (Fig. 7A). We obtained the same average match in a comparison of the exuvial and flagellar columns with the exception of one unique alignment for each pair, where matching reached between 72–85% (Fig. 7B). Such matchpoints were not found if the exuvium was compared with the contralateral flagellum of the same animal (Fig. 7C) nor were they found if the exuvial column was moved proximally past the flagellar column.

These comparisons suggest that new ORNs are being differentiated at the proximal end of the aesthetasc array. This is supported by the presence of developing clusters of ORNs and aesthetascs that are visible beneath the proximal cuticle of fixed and cleared antennules from intermoult animals (Fig. 9). The possibility that older distal segments are being shed at moulting was confirmed by finding such segments remaining within the exuvia. When matched with the new flagellum, this part of the exuvium clearly protruded beyond the new distal tip of the flagellum.
Discussion

The results of this study show that the distal end of the lateral flagellum of the antennule is the first to differentiate and produce the olfactory receptor neurones and their sensilla. Segments are then added at each moult to the lateral flagellum at its proximal end. The flagellum continues to lengthen without losing the receptors at the distal tip (unless damaged) by adding aesthetasc-bearing segments at the proximal end of the receptor array. It would appear that the chances of losing the distal tip of the flagellum increase with body size because the characteristic numerical pattern of the distal end of the postembryonic flagellum is seldom seen in animals larger than approximately 8 mm carapace length. Older animals shed the distal segments with the exuvium. New segments, in excess of those that are shed, are recruited at the proximal end of the receptor array, and the flagellum gradually increases in length. Matching the exuvium with the newly moulted flagellum provides convincing evidence for the above. The near, but not perfect, match provides us with further insights into the way in which aesthetasc sensilla are added to the flagellum. In the smaller animals, no match could be found when aesthetasc numbers were compared, but in some larger animals the aesthetasc hairs provided the highest percentage match of all receptors compared. The explanation for this may lie in the gradual increase in number of aesthetasc sensilla per segment that is correlated with the increase in the body size (Table 1). Small animals rarely have rows of four or five aesthetascs, whereas this is common in the larger animals. The mismatch between aesthetasc numbers on the exuvium and flagellum in smaller animals, when these are aligned for the guard hairs, could therefore be produced by a local increase in ORN and aesthetasc numbers. An examination of the mismatching segments proved this to be the case. Thus, in the early stages of the animal’s life, ORNs and aesthetascs are added along the entire length of the array as well as at the proximal end. This effect is enhanced by the production of segments at the proximal end of the array that have the full complement of four (or five) sensilla and the gradual loss of the distal segments that carry smaller numbers. In the older animals, a local increase in numbers of aesthetasc sensilla appears to be rare.

The imperfect match between the guard hairs is also a good indication that changes can still occur along the length of the flagellum. Where mismatches occurred at the matchpoint, they were found to be caused by an increase in the number of hairs present.

The comparison of the left exuvium with the right newly moulted flagellum sometimes produced matching that was...
One aspect of the flagellar growth remains unclear. The base of the flagellum in mature animals rests on the ischiopodite, and the annuli of the proximal half of the flagellum carry no aesthetasc hairs. Towards the proximal end of the aesthetasc receptor array, feather hairs are absent from the ventral surface of the segments. The feather hairs are gradually restricted to the dorsal surface of the flagellum and are absent over the proximal aesthetasc-bearing segments. From this observation and the presence of developing ORNs and aesthetascs beneath the cuticle of the undifferentiated segments, it would appear that the segments of the flagella are not being produced de novo at the proximal end of the chemoreceptor array but are being gradually transformed into chemoreceptive segments. How this is controlled provides us with an interesting question which may be approached through the ability of the antennules to regenerate after damage.

Given that the chemoreceptors are being shed from the distal end of the flagellum and that new receptors are being formed at the proximal end of the array, we are able to calculate an approximate rate of receptor turnover (in this case equivalent to the loss of the distal receptors) as, in all but the largest animals, more receptors will be added than are lost. Receptor turnover in crayfish can only occur at moulting, and the frequency of moulting is dependent on the amount of food they obtain, the temperature of the water in which they live and their size. Nevertheless, matching the exuvia of a 55 mm carapace length animal with its newly moulted flagellum showed it to have lost seven distal segments carrying 23 aesthetasc sensilla. The total aesthetasc count on the exuvia amounted to 150, whereas the new flagellum carried 180, i.e. a gain of 30 sensilla. Twenty-three of these can be considered as replacements, leaving a net gain in this animal of seven sensilla. The approximate total turnover of ORNs lies in the region of 2300.

The serial replacement of the ORNs along the antennule of the crayfish differs markedly from the replacement of the ORNs in vertebrates in a way that may have important consequences for the central organization of the olfactory systems of the two groups of animals. Recently, Breer and his colleagues have shown that the membranes of ORNs in
discrete areas within the olfactory epithelia in rats contain the same kind of receptor molecules and so are sensitive to the same kinds of odours (Breer, 1993, 1994; Strotmann et al. 1994). ORNs in these areas must be continually replaced with others of the same kind, otherwise the demonstrated specificity of these areas would soon vanish. The rat olfactory epithelium therefore exhibits a certain spatial organization that reflects odour selectivity which could be centrally preserved because axons from the same areas of the epithelium are likely to end in close proximity to one another in the olfactory bulb.

In contrast, the spatial concentration of sensilla with a specific odour sensitivity on the crayfish antennule would result in the sensitivity to that particular odour being severely attenuated when the animal moulted, unless the lost portion was simultaneously replaced with receptors of the same sensitivity. It is more likely that each row of aesthetasc sensilla has the entire spectrum of odour sensitivities that the animal needs and that the central projections from each row will extend over the entire olfactory lobe. Radioactive leucine tracing of the central projections of the aesthetasc sensilla from a single segment has shown this to be the case (Mellon, 1990). This notion is also supported by physiological studies of lobsters where single rows of aesthetasc sensilla and even single sensilla have the entire spectrum of odour sensitivities (Spencer, 1986).

References


