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ECDYSIS OF DECAPOD CRUSTACEANS IS ASSOCIATED WITH A DRAMATIC RELEASE OF CRUSTACEAN CARDIOACTIVE PEPTIDE INTO THE HAEMOLYMPH

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Summary

On the basis of detailed analyses of morphological characteristics and behavioural events associated with ecdysis in a crab (*Carcinus maenas*) and a crayfish (*Orconectes limosus*), a comprehensive substaging system has been introduced for the ecdysis stage of the moult cycle of these decapod crustaceans. In a remarkably similar stereotyped ecdysis sequence in both species, a passive phase of water uptake starting with bulging and rupture of thoracoabdominal exoskeletal junctions is followed by an active phase showing distinct behavioural changes involved in the shedding of the head appendages, abdomen and pereiopods. Together with an enzyme immunoassay for crustacean cardioactive peptide (CCAP), the substaging has been used to demonstrate a large, rapid and reproducible peak in haemolymph CCAP levels (increases of approximately 30-fold in the crab and more than 100-fold in the crayfish compared with intermoult titres) during the later stages of active ecdysis. We suggest that the release of CCAP (accumulated in late premoult) from the crab pericardial organs or the crayfish ventral nerve cord accounts for many of the changes in behaviour and physiology seen during ecdysis and that this neurohormone is likely to be of critical importance in crustaceans and other arthropods.

Key words: ecdysis, crustacean, crab, *Carcinus maenas*, crayfish, *Orconectes limosus*, crustacean cardioactive peptide, moult, behaviour.

Introduction

Moulting is a prerequisite for development and growth in crustaceans and other arthropods. Among the different moult stages, the stage of ecdysis comprising the actual exuviation process is the shortest stage, but the most crucial step in the life cycle of any of these invertebrates, because death is inevitable if it cannot be completed successfully. The ecdysis of a freshwater crayfish and some aspects of the stereotyped behaviour involved were described in detail at the beginning of the eighteenth century (Réaumur, 1712, 1718a,b) and later refined to some extent for crustaceans including crayfish (Huxley, 1880; Aiken, 1968; Aiken and Waddy, 1987), crabs (Vitzou, 1882; Drach, 1939; Guyselman, 1953; Passano, 1960), prawns (Jefferies, 1964), lobsters (Travis, 1954; Aiken, 1980; Waddy et al., 1995) and isopods, the latter showing a complex biphasic ecdysis (Carlisle, 1956). Most authors have divided the procedures leading to the shedding of the old exoskeleton into a passive phase, mainly characterised by significant water uptake, followed by an active phase of exuviation behaviour. Because of the short duration of this process, only one author has so far tried to subdivide this important stage of the crustacean moult cycle in the crayfish *Orconectes virilis* but merely on the basis of moult mortalities (Aiken, 1968).

Several reports have dealt with the physiological events that accompany crustacean ecdysis. In crabs and lobsters, ecdysis involves profound changes in the rate of absorption of several ions, especially Mg^{2+} and Ca^{2+} (Robertson, 1960; Wheatly, 1985), connected with the rapid water uptake initiated in late premoult stage D_4 (Baumberger and Olmsted, 1928; Dandrifosse, 1966; Dall and Smith, 1978; Mykles, 1980; Neufeld and Cameron, 1994). A transient increase in hydrostatic haemolymph pressure (deFur et al., 1985), a decrease in the rate of oxygen consumption and an increase in the rate of carbon dioxide and lactate production also occur at this time (Lewis and Haefner, 1976; Mangum et al., 1985b; Mangum, 1992; Clemens et al., 1999). There is only one report of the electrical activities of the heart, stomach and skeletal muscles in a spiny lobster (Kuramoto, 1993). However, surprisingly little is known about the mechanisms controlling the initiation and maintenance of the crustacean ecdysis process and the stereotyped behaviour patterns involved.

The induction of moult and a successful ecdysis in crayfish has been suggested to be dependent upon photophase length, upon the balanced concentrations of a moulting hormone (ecdysteroids) and a moult-inhibiting hormone (MIH) (Aiken, 1969) and upon water temperature (Westin and Gydemo,

1986). A currently accepted model of moult control in crustaceans involves the inhibitory neuroendocrine regulation of Y-organ ecdysteroid biosynthesis in intermoult by MIHs from the X-organ/sinus gland (XOSG) neurosecretory system of the eyestalk. These neuropeptides are members of a large peptide family also including crustacean hyperglycaemic hormones (CHHs) and gonad/vitellogenesis-inhibiting hormones (GIHs/VIHs) (for reviews, see Keller, 1992; Chang et al., 1993; Webster, 1998). However, almost nothing is known about the endocrine regulatory principles governing the ecdysis process. The first preliminary evidence for an involvement of the neuropeptide crustacean cardioactive peptide (CCAP) in crustacean ecdysis was reported by Johnen et al. (1995), who demonstrated that CCAP levels in the ventral nervous system of the isopod *Oniscus asellus* are elevated shortly before and during ecdysis. Furthermore, recent investigations have demonstrated a large transient increase in levels of a CHH in the haemolymph of shore crabs, *Carcinus maenas*, associated with ecdysis. This CHH is identical to that known from the XOSG system, but is released from hitherto unknown gut paraneurons only shortly before, during and after ecdysis and obviously drives water uptake during this process (Chung et al., 1999).

Compared with crustaceans, ecdysis has been investigated for more than 30 years in insects, resulting in a much deeper understanding of the endocrine regulatory principles. These involve an eclosion hormone (EH), ecdysis-triggering hormone (ETH) and crustacean cardioactive peptide (CCAP), studied principally in the sphingiid moth *Manduca sexta* and its developmental stages (for reviews, see Hesterlee and Morton, 1996; Truman et al., 1998). A central role for CCAP in initiating ecdysis motor programmes within the central nervous system under the influence of ETH and EH has recently been demonstrated (Gammie and Truman, 1997a,b, 1999). Although in *M. sexta*, during all developmental stages, some CCAP neurons in the ventral nerve cord project into peripheral neurohaemal release sites (Davis et al., 1993), almost nothing is known about the putative neurohormonal roles of CCAP and/or other cardioactive peptides (CAPs) during ecdysis with the exception of a proposed role for CAPs in heartbeat control during wing-spreading behaviour (Tublitz and Truman, 1985b).

In this paper, we describe the natural ecdysis process of two decapod crustacean species, the shore crab *Carcinus maenas* and the crayfish *Orconectes limosus*, in greater detail than before and introduce a novel nomenclature for subdivisions of the stage of ecdysis (stage E; Drach, 1939; Drach and Tchernigovtzeff, 1967) in the decapod moult cycle on the basis of morphological and behavioural criteria applied to living specimens. This was necessary since a previously proposed substaging of E (Aiken, 1968) could not be considered as a useful basis for our studies because it merely reflects results obtained artificially after manipulations during premoult. As has been discussed previously (Rao et al., 1973), these stages therefore represent the results of unnatural changes leading to distinct forms of death in stage E obviously caused by certain developmental deficiencies attained during premoult, rather than describing any of the natural processes during ecdysis in living animals. Furthermore, we show using a novel and evaluated assay system that crustacean ecdysis is invariably associated with the release of large quantities of CCAP into the haemolymph during ecdysis and that this release can be set into the behavioural context of active ecdysis behaviour. We show that CCAP is released from the crayfish ventral nerve cord, which is known to contain large areas of perineural release sites for CCAP. These are distributed along the entire dorsal perineural sheath of the ventral nerve cord, including the segmental nerve roots, and originate from identified serially homologous CCAP-immunoreactive neurosecretory neurons (Trube et al., 1994).

Materials and methods

Animals, moult staging and moulting behaviour

Mature adults of medium-sized (carapace width 5–6 cm) shore crabs, *Carcinus maenas* L., of all stages, especially those in the premoult stages (so-called ‘peeler’ crabs), were collected in July and August (1997 and 1998) from the shore off the Isle of Anglesey, UK. The animals were maintained in individual culture tanks connected to a recirculating seawater system at 12–15 °C under a light:dark photoperiod of 16 h:8 h.

Adult specimens of the crayfish *Orconectes limosus* Rafinesque, 1871 were caught from the river Havel in Berlin and obtained from a local fisherman. Animals were maintained under running tap water in large aquaria at a water temperature of 12–15 °C and were fed pelleted cat food twice a week. These stock aquaria received a light:dark photoperiod of 12 h:12 h. Mature premoult crayfish with a rostrum–telson length of 7–9 cm were transferred into individual culture tanks under a light:dark photoperiod of 16 h:8 h at room temperature (18–22 °C).

The moult stages of premoult crabs were determined by investigation of the setal development of the basipodite of the second maxilla, by morphological criteria, such as carapace and epimeral suture rigidities, and by behavioural variables. Moult staging of premoult crayfish was carried out using a setal index according to Aiken and Waddy (1987). Some further morphological variables are introduced in the present study (see below). In general, the moult stages referred to the staging system first introduced by Drach (1939) and further modified for different crustaceans by several authors (for a review, see Aiken and Waddy, 1987). In the present report, a substaging system of stage E has been defined by the use of morphological and especially behavioural variables (see Results) and following the three criteria of Drach (1939) for the usefulness of a such staging system; i.e. permitting rapid stage recognition of the living animal, applicability to animals of different sizes and to different species of decapod crustaceans, and accounting for transformations without placing undue emphasis upon ecdysial staging based upon time intervals.

Crab ecdysis was documented on colour slide film. In the case of crayfish ecdysis, single video frames were taken with

a Kappa no. CF 15/2 video camera connected to a Panasonic NV-HS1000 video recorder. The images were digitised using a Miro Video DC 30 frame grabber card in a personal computer and analysed using image-processing software (Adobe Premiere 4.2, Corel Photopaint 7.0).

Purification of haemolymph samples

Haemolymph was purified by a method developed to give the highest recoveries and the most consistent results. Samples (200–300 µl) of haemolymph were taken from the ventral abdominal sinus (*Orconectes limosus*) or the hypobranchial sinus (*Carcinus maenas*) and snap-frozen in liquid nitrogen. Prior to purification, samples were gently thawed and centrifuged (2500 ***g***, 5 min, 4 °C). A defined volume of the supernatant was then slowly (approximately 1 ml min^{-1}) applied to a Sep-Pak C_{18} cartridge (Waters) preconditioned with 10 ml of 60 % acetonitrile and equilibrated with 10 ml of water, both at a flow rate of 10 ml min^{-1}. After loading of the sample, the cartridge was washed with 10 ml of water followed by elution of the peptide fraction with 3 ml of 60 % acetonitrile, again at a slow flow rate (1 ml min^{-1}); the first 0.5 ml (void volume) was discarded. The eluate was collected into a Minisorb tube (NUNC Gibco), dried in a vacuum centrifuge (Speed Vac, Savant) and redissolved in 125–200 µl of assay buffer (PBST) containing (in mmol l^{-1}): NaCl, 138; Na_2HPO_4, 11.25; KCl, 2.68; KH_2PO_4, 1.76; Tween 20, 0.1 % v/v; pH 7.4). The recovery of CCAP after Sep-Pak purification was determined by adding an ^{125}I-radiolabelled synthetic CCAP analogue P*Y*CNAFTGC-NH_2 (Y^2-CCAP; see below) to the haemolymph samples. Since the hormone contents of the haemolymph varied over a wide range at different moult stages, every sample was assayed in a dilution series to find the best fit within the linear range of the assay.

CCAP enzyme immunoassay

A highly sensitive competitive enzyme immunoassay (EIA) has been developed for CCAP by modifying a principle described previously (Kingan, 1989). Synthetic CCAP was coupled to human serum albumin (HSA, Sigma) using 1-ethyl-3,3′-diaminopropyl-carbodiimide (EDC, Sigma) at a molar ratio of CCAP:HSA:EDC of 50:1:560 according to Skowsky and Fisher (1972). This CCAP–HSA conjugate was diluted 1:1500 in phosphate buffer (PB, 0.1 mol l^{-1}, pH 8.0) and coated overnight (4 °C, humid atmosphere) onto 96-well microtitre plates (NUNC, Maxisorp F16). After three washing steps in PB (0.01 mol l^{-1}, pH 8.0), the plates were blocked with normal goat serum (NGS; 1 % in PB, 0.1 mol l^{-1}, pH 8.0) for 2 h at room temperature. The blocking buffer was poured out, and the plates were either used immediately or stored for up to 2 weeks at 4 °C. A standard curve was prepared in assay buffer (PBST) in the range 0–400 fmol of CCAP per well (50 µl). Thereafter, 50 µl per well of a primary antiserum against CCAP (code 1TB; Dircksen and Keller, 1988) diluted in PBST was added to standards and samples at a final antiserum concentration of 1:3000, and the plates were incubated overnight at 4 °C in a sealed humid chamber. After three washes in PBST, 100 µl per well of an enzyme-labelled secondary antibody (sheep anti-rabbit IgG–peroxidase conjugate, Boehringer) was added at a concentration of 1:3500 diluted in PBST, and the plates were incubated for 1 h at room temperature in a humid chamber. Finally, after three washes in PBST, 100 µl of the substrate solution containing 0.2 µl ml^{-1} 30 % hydrogen peroxide and 0.4 mg ml^{-1} 2,2′-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid (Sigma) in citrate buffer, pH 4.0, was added, and the mixture was incubated for 1 h at room temperature. The optical density of the reaction products was then measured with an EIA reader (MK II Multiscan Plus, Flow Laboratories) at 405 nm. The concentrations of standards and samples were evaluated using a connected computing system (software version EIA3, Flow Laboratories). For statistical evaluations, a computerised program was used (Microsoft Excel version 5.0).

High-performance liquid chromatography (HPLC)

The presence of CCAP in the haemolymph and in extracts of the pericardial organs of crabs in different moult stages, dissected under ice-chilled crab saline (Webster, 1986), was identified by HPLC analysis and subsequent CCAP–EIA analysis of every fraction. Haemolymph collected from intermoult animals (stage C_4, 20 ml) was directly compared with haemolymph from animals that were in the active part of ecdysis (stage E_{30}–E_{100}, 20 ml). The samples were collected from the hypobranchial sinus and immediately frozen in liquid nitrogen. Prior to HPLC purification, the haemolymph was gently thawed and centrifuged for 5 min at 2700 ***g*** (at 4 °C) to remove remaining cells and debris. The supernatant was prepurified using Sep-Pak cartridges (2 ml per cartridge) preconditioned with 10 ml of isopropanol (100 %) followed by equilibration with 10 ml of water and eluted with 3 ml of 40 % isopropanol. The eluates were pooled and dried in a vacuum centrifuge, and the resulting pellet was redissolved in 200 µl of 2 mol l^{-1} acetic acid prior to chromatography under the following conditions. A linear gradient of 30 % to 80 % solvent B [solvent A: 0.11 % trifluoroacetic acid (TFA); solvent B: 0.1 % TFA in 60 % acetonitrile] over 60 min was used to elute a phenyl column (Waters µBondapak, 4.6 mm×250 mm) at a flow rate of 1 ml min^{-1}. Fractions of 1 ml were collected automatically, dried, redissolved in PBST and assayed for CCAP immunoreactivity in a dilution series. A standard of 10 pmol of synthetic CCAP in 2 mol l^{-1} acetic acid was chromatographed immediately after the sample under the same conditions.

Gel filtration of crayfish haemolymph

Haemolymph from crayfish that had just completed ecdysis (stage E_{100}) was compared with haemolymph from intermoult animals (stage C_4) using gel filtration, and every fraction was assayed for CCAP immunoreactivity by CCAP–EIA. Haemolymph was collected from the ventral abdominal sinus and immediately frozen in liquid nitrogen. Prior to gel filtration, the sample was thawed, centrifuged (2000 ***g***, 5 min, 4 °C) and the supernatant diluted 2:1 with an anticoagulant

buffer (PBS containing 1 % glycine ethyl ester). The diluted haemolymph was chromatographed at 4 °C on a 90 cm×0.6 cm column packed with Sephadex G-50 superfine (Pharmacia) using 0.1 mol l^{-1} PBS, pH 7.4, as eluant. Fractions of 400 µl were collected automatically at intervals of 15 min, and their optical density was measured at 220 nm. Subsequently, every fraction was assayed in duplicate for CCAP immunoreactivity.

Iodination of Y^2-CCAP and injection into crabs

Small quantities (approximately 0.25 nmol) of synthetic Y^2-CCAP were iodinated with 9.25 MBq of $Na^{125}I$ (Amersham) using chloramine-T (Bolton, 1989). The purification of the radiolabelled peptide was carried out with a Sep-Pak cartridge previously conditioned with isopropanol and equilibrated with water. The labelled peptide was eluted with 3 ml of 40 % isopropanol. A specific activity of approximately 26 TBq $mmol^{-1}$ was obtained.

To determine the half-life of the hormone within the haemolymph, crabs were injected with radiolabelled Y^2-CCAP in crab saline (Webster, 1986) into the coxopodite of the third leg, and haemolymph samples (100 µl) were taken from the hypobranchial sinus at different intervals (2, 5, 10, 20 and 30 min). After having taken the last haemolymph sample, the animals were anaesthetised on ice, killed by cutting the connectives between the cerebral and thoracic ganglia, and the gills and heart were dissected out. Haemolymph samples, gills and heart tissue were examined for radiolabelled Y^2-CCAP using a gamma scintillation counter (Wallac 1470).

In vitro *release experiments*

Entire ventral nerve cords without brains were carefully dissected in ice-cold standard saline (in mmol l^{-1}: NaCl, 205; KCl, 5.4; $CaCl_2$, 15.3; $MgCl_2$, 2.5; maleic acid, 5.0; Trizma base, 5.0; pH 7.4; modified after Van Harreveld, 1936). The ganglia were partially desheathed on the ventral side and incubated in tissue-culture plates (Linbro) at room temperature in a volume of 1 ml of different salines for 5 or 10 min. The standard procedure included several washing steps in Ca^{2+}-free washing saline (WS; standard saline without Ca^{2+}) interrupted by control steps in two different control salines [control saline 1 (CS1): free of Ca^{2+}, 10×KCl (54 mmol l^{-1}); control saline 2 (CS2): standard saline]. The nervous system was depolarized with releasing saline (RS) in which the K^+ content had been increased tenfold in the presence of Ca^{2+} (54 mmol l^{-1} KCl, 15.3 mmol l^{-1} $CaCl_2$). Incubations with control or releasing saline were always preceded and followed by at least two steps in washing saline. High-K^+ salines (CS1, RS) were depleted of the same amount of Na^+ (NaCl), and Ca^{2+}-free salines (WS, CS1) were supplemented with the same amount of Mg^{2+} ($MgCl_2$) to maintain the same osmolarity.

All samples were frozen immediately after the incubation. Since Ca^{2+} obviously interferes with the phosphate buffer system in the CCAP–EIA, all samples had to be depleted of Ca^{2+}. This was achieved either by Ca^{2+} precipitation using ice-cold phosphate buffer, followed by centrifugation (12 000 ***g***, 5 min, 4 °C), or by Sep-Pak purification. The conditioning, equilibration and elution of the Sep-Pak cartridges were carried out as described above for the haemolymph samples. The eluate was dried in a vacuum centrifuge, redissolved in assay buffer (PBST, pH 7.4) and tested for released CCAP in a dilution series.

Results

The ecdysis (E) stage of the green shore crab is differentiated into substages by the use of overt morphological and behavioural variables that are characteristic of the respective stages and are also applicable to the American crayfish, as summarised in Fig. 1. The proposed nomenclature (E_0–E_{100}) indicates the percentage of the whole ecdysis process. These percentages cannot strictly be correlated with the temporal pattern of ecdysis since the duration of a single behavioural event can vary considerably among the specimens. However, we observed that the sequences of appearance of distinct morphological characteristics and behavioural elements were absolutely constant among all specimens examined (N>15 for both species). Two major parts of the ecdysis process can be distinguished, the passive and the active parts. Whereas the passive phase (E_0–E_{30}) is mainly characterised by extensive water uptake resulting in body swelling and by pronounced inactivity of the animals, the active phase (E_{30}–E_{100}) comprises the final active and stereotyped exuviation behaviour patterns that include several rhythmic muscle movements in a fixed sequence with regard to their appearance in different parts of the body. This substaging allowed reproducible and substage-refined haemolymph sampling to unravel the temporal patterns of CCAP titres during ecdysis.

Ecdysis of the shore crab

The duration of a complete ecdysis (passive and active) of crabs varies from 45 to 90 min, because the animal can prolong or even interrupt the passive phase, e.g. when disturbed by a potential predator, or, as is occasionally seen in the case of male crabs, when mating behaviour is preferred in the presence of a freshly moulted female of stage A or B. The entire ecdysis usually takes approximately 60 min, during which the passive phase lasts 45–50 min and the active phase takes 10–15 min in males and 5–10 min in females. The transition between the last premoult stage D_4 and stage E_0 is characterised by the widening from back to front of the pleural suture, which has opened to a narrow gap (finally up to approximately 1 mm) several hours before the ecdysis as a result of the beginning of water uptake in stage D_4 (Fig. 1). At a final gap size of 2–3 mm (stage E_5), the thoracoabdominal intertergal membrane ruptures, and continuous lifting of the old carapace starts in response to more extensive water uptake by the animal (Figs 1, 2A,B). Whilst this lifting of the back of the carapace continues beyond an angle of approximately 30 ° to the longitudinal axis, active ecdysis starts with the so-called ‘wiggling’ behaviour (Fig. 1) when the lateralmost spines of the new dark green

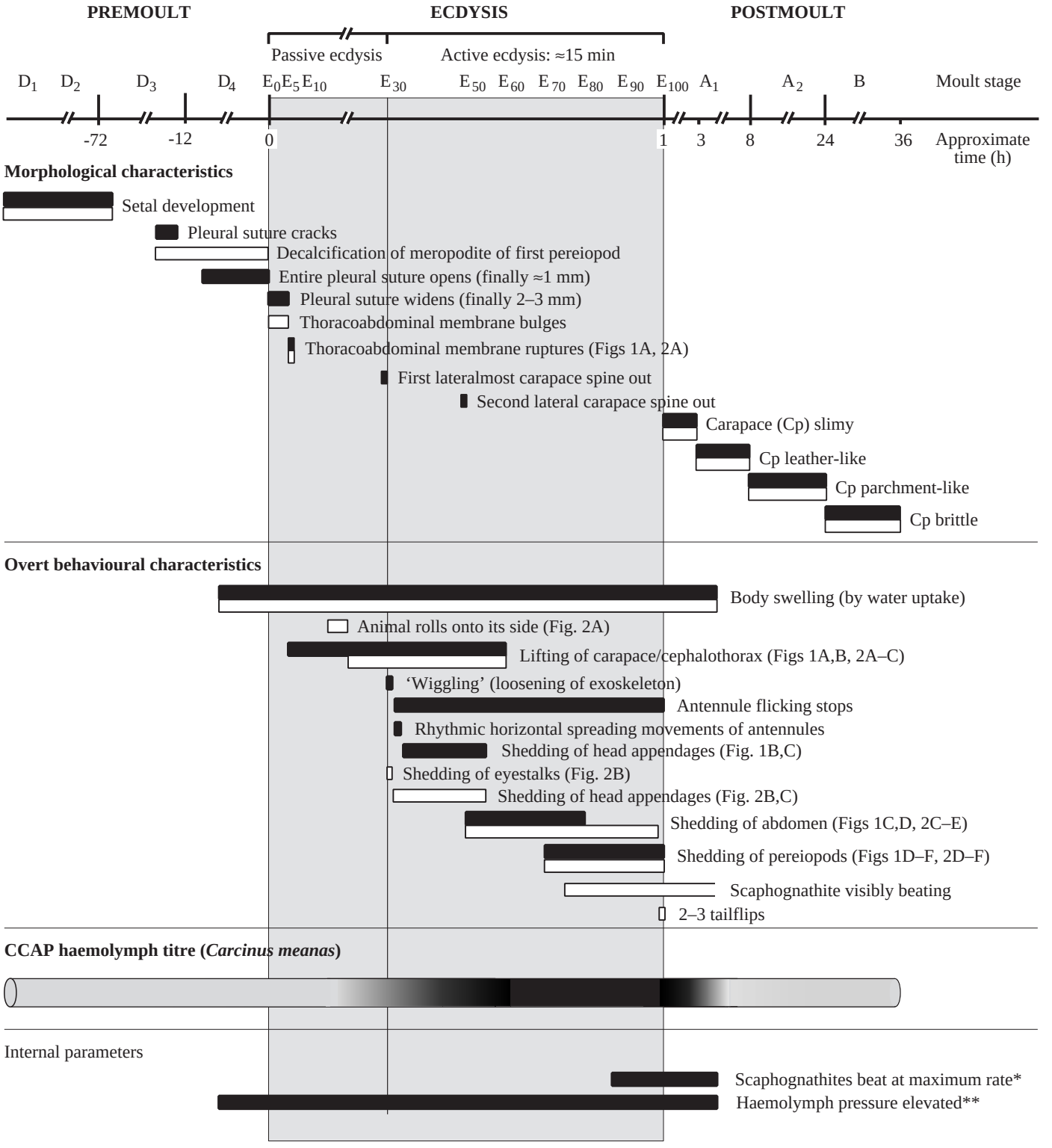

Fig. 1. Diagram summarising and comparing the overt morphological and behavioural characteristics during crab and crayfish moult stages (according to Drach, 1939) and introducing novel subdivisions for stage E (ecdysis) of the moult cycle. Horizontal bars represent periods around and within the passive and active ecdysis phases when particular characteristics occur; filled bars, characteristics of crab ecdysis; open bars, characteristics of crayfish ecdysis (see text for details). The sketch of crustacean cardioactive peptide (CCAP) haemolymph content during crab ecdysis shows accumulation during active ecdysis reaching a maximum by the end of ecdysis (E_{100}). *Passano (1960); **deFur et al. (1985).

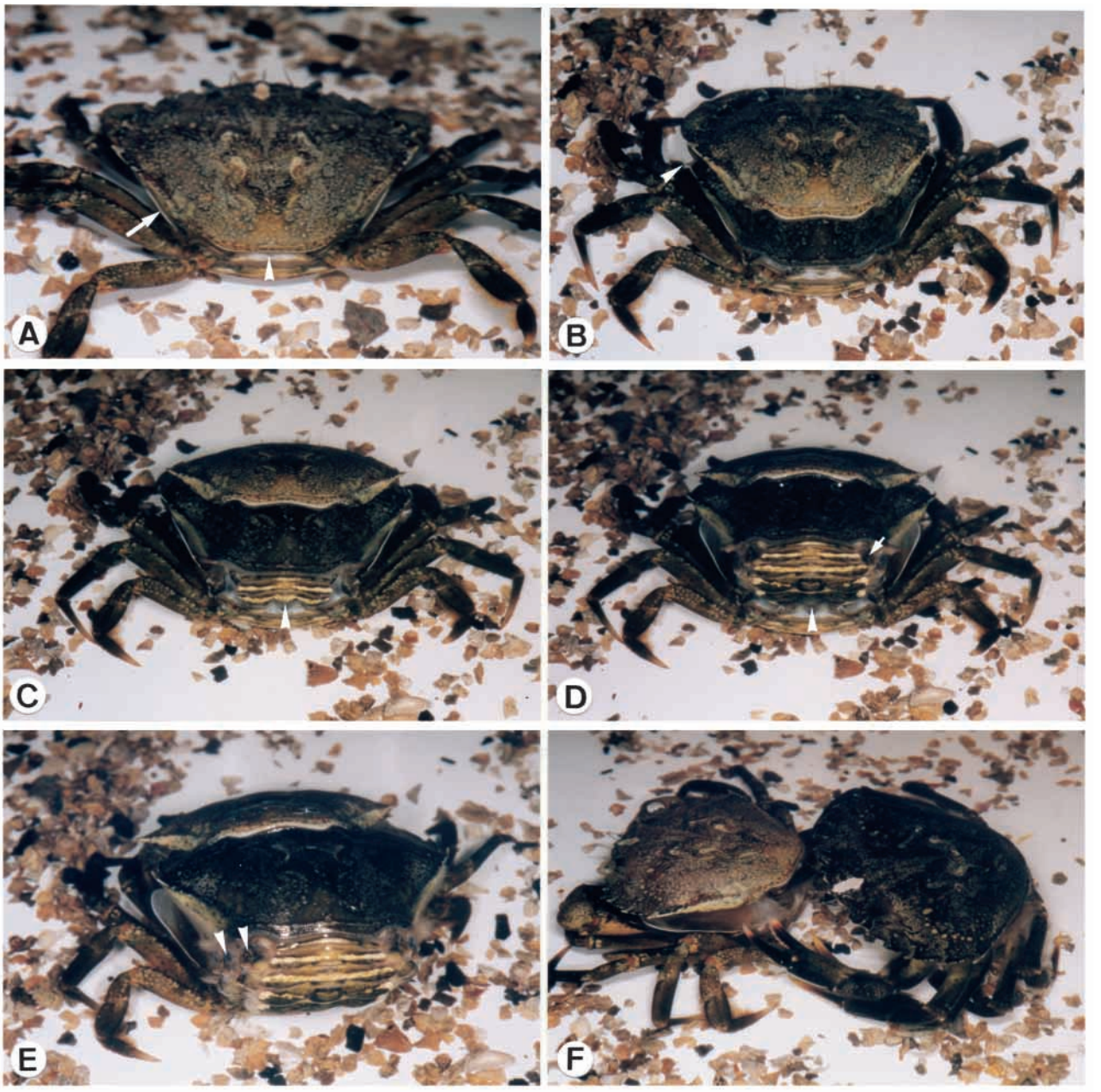

Fig. 2. The ecdysis behaviour of a female green shore crab *Carcinus maenas* (carapace width 6 cm). (A) Passive ecdysis in stages E_0–E_{10} characterised by the widening of the pleural suture (arrow) and the rupture of the thoracoabdominal membrane (arrowhead). (B) Stage E_{30}: start of the active phase with shedding of the head appendages, after the lateralmost spines (arrowhead) of the new carapace become visible. (C) Head shedding in stage E_{50}–E_{60} directly followed by abdominal shedding (arrowhead; third abdominal segment). (D) Stage E_{70}, shedding of the pereiopods has started (small arrow; coxopodite of fifth leg) after the fifth abdominal segment (arrowhead) is withdrawn. (E) Stage E_{80}–E_{90}, pereiopodal shedding in the last phase of active ecdysis, abdominal shedding has finished (arrowheads; coxopodites of fourth and fifth leg). (F) Stage E_{100}, ecdysis is finished when the chelae are completely withdrawn.

carapace become clearly visible and emerge below the old carapace (Fig. 2B; stage E_{30}). The wiggling movements affect all appendages of the body for a very short period (approximately 20 s) and obviously assist the general loosening of the whole exoskeleton. Within a few minutes of the wiggling movement, the active shedding process of the head appendages clearly starts with unique and rhythmic horizontal spreading movements of the antennules, which halt their previously regular perpendicular flicking movements at this time. Head shedding consists of the shedding of the antennae, the eyestalks and the mouthparts, including the scaphognathites, thus depriving the animal of sensory inputs and of an oxygen supply from the water during these stages (Fig. 2B,C; E_{30}–E_{50}). This is almost directly followed by shedding of the abdomen, which

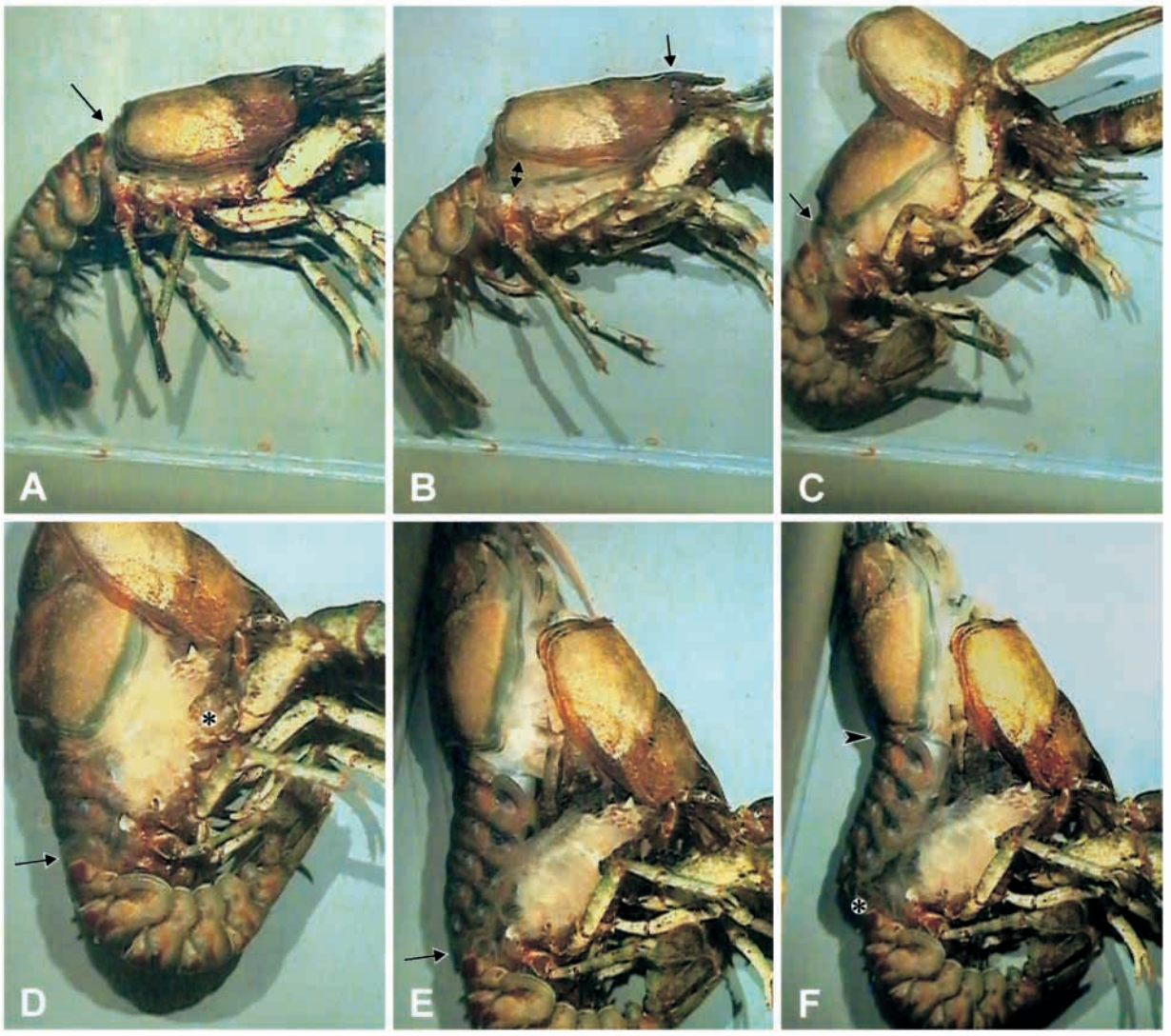

Fig. 3. Video snap shots showing the ecdysis of an adult male American crayfish *Orconectes limosus* (rostrum–telson length 7 cm). (A) Thoracoabdominal membrane rupture (arrow) during the passive phase has passed, and the animal has rolled onto its side (E_{10}). (B) Carapace lifting (E_{30}; double-headed arrow) and initiation of the active phase by shedding of the head appendages, first visible by the retraction of the eyestalks (arrow). (C) Abdominal shedding starting at the end of shedding of the head appendages (E_{50}; arrow; first abdominal segment). (D) Shedding of the pereiopods starting with peristaltic movements of the coxopodites (E_{70}; arrow, fourth abdominal segment; asterisk, coxopodite of the right cheliped) after half of the abdomen has been withdrawn. (E) Parallel performance of abdominal and pereiopodal shedding (E_{80}–E_{90}; arrow, fifth abdominal segment). (F) Withdrawal of the last abdominal segments (uropods and telson; E_{100}; asterisk, uropod) when muscle tension between the cephalothorax and abdomen builds up (arrowhead) shortly before 2–3 tailflips complete the ecdysis.

covers the stages E_{50}–E_{80} and is characterised by peristaltic contractions of the abdominal body musculature (Fig. 2C,D). Only after the third abdominal segment is withdrawn (Fig. 2C) does the animal begin characteristic contractions of the thoracic coxopodites and basipodites. These contractions, which always alternate between the two sides (duration approximately 5–6 s per contraction), mark the beginning of pereiopodal shedding, which is performed during stages E_{70}–E_{100} (Fig. 2D–F). The alternating coxopodite contractions are performed regardless of whether the corresponding leg exists. Abdominal shedding is terminated by stage E_{80} when pereiopodal shedding exhibits the strongest muscle movements (Fig. 2E). The entire ecdysis is finished when the chelae are completely removed from the old exuvia (stage E_{100}; Fig. 2F). Immediately after exuviation, the animal continues to take up water and expands to its final size within the first 2–3 h after ecdysis during stage A_1.

Ecdysis of crayfish

The behavioural pattern during ecdysis of the crayfish is similar to that of the crab with only minor differences. The onset of passive ecdysis is difficult to determine because water uptake commences in stage D_4 and continues throughout passive ecdysis but is less prominent than in the

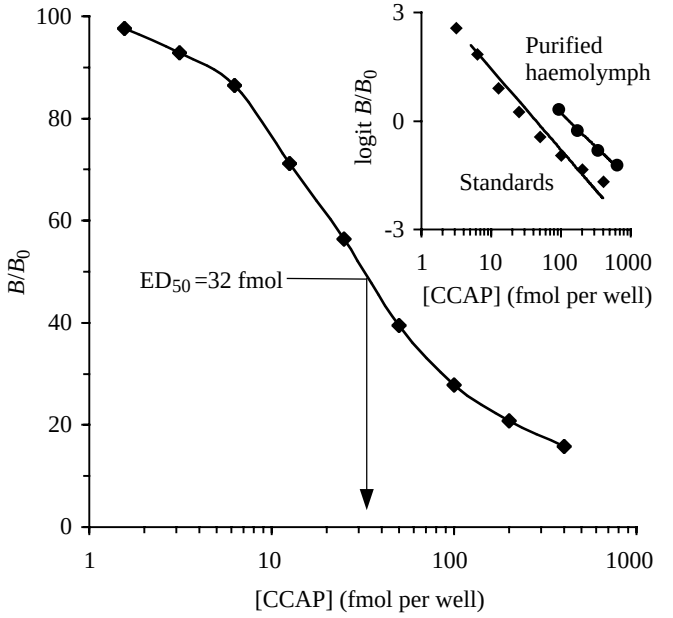

Fig. 4. Typical standard curve of the competitive enzyme immunoassay (EIA) for the detection of crustacean cardioactive peptide (CCAP) showing an ED_{50} of approximately 32 fmol (the detection limit of the assay being repeatedly below 5 fmol), where B/B_0 is the percentage bound (B; not displaced by free CCAP) *versus* maximum bound (B_0) antibody. The inset shows a logit/log plot analysis of Sep-Pak-purified ecdysis haemolymph samples in serial dilution measured in the same assay. Note that the curves for CCAP standards and CCAP-immunoreactive haemolymph samples are parallel (regression lines: standards, $y=-0.89\ln x+3.3$, $r^2=0.977$; for haemolymph, $y=-0.80\ln x+3.9$, $r^2=0.992$), indicative of similar competition by both substances in the assay. For clarity, the graph of the dilution series of haemolymph samples has been shifted to the right by a factor of 4.

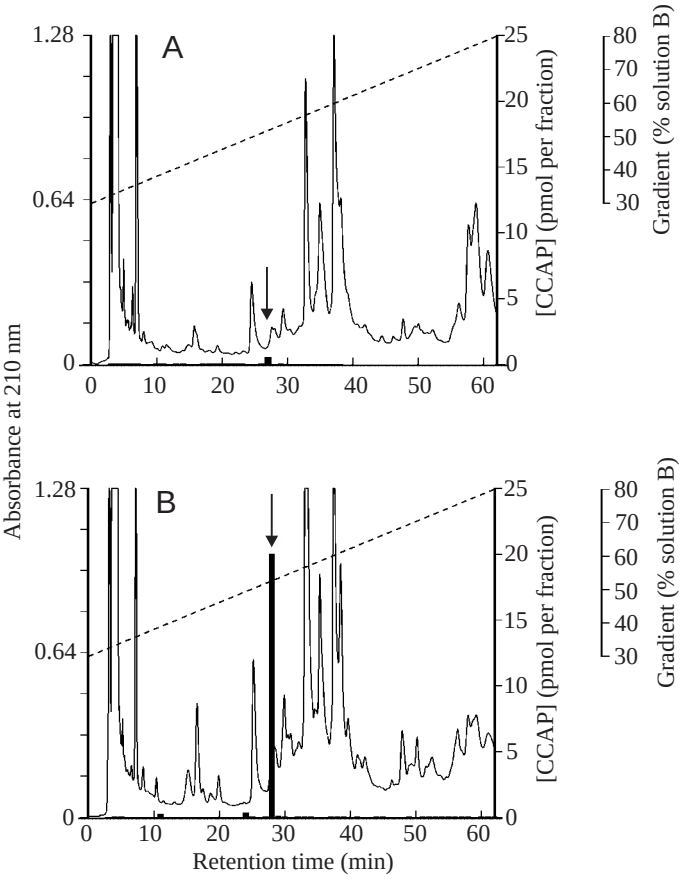

Fig. 5. (A,B) HPLC fractionation (1 ml min^{-1}) and subsequent assay for crustacean cardioactive peptide (CCAP) immunoreactivity of each fraction by competitive enzyme immunoassay (EIA) of intermoult haemolymph (A; 20 ml collected from stage C_4 animals) and of ecdysis haemolymph (B; 20 ml collected during active ecdysis in stages E_{80}–E_{100}) of the shore crab *Carcinus maenas* showing the identical elution time of the synthetic peptide and the immunoreactive fractions of the haemolymph (arrows). Note that the CCAP haemolymph titre amounts to approximately 1 pmol ml^{-1} (10^{-9} mol l^{-1}) at ecdysis compared with 30 fmol ml^{-1} (3×10^{-11} mol l^{-1}) in intermoult.

crab. However, bulging of the thoracoabdominal intertergal membrane marks the point of transition from stage D_4 to passive ecdysis, which has definitely started when the thoracoabdominal membrane is ruptured (Fig. 1; stage E_5). The animal finally rolls onto its side (stage E_{10}; Figs 1, 3A) and continues with the elevation of the posterior margin of the carapace, thereby revealing the homogeneously dark brownish new carapace (Fig. 3B). The onset of active ecdysis starts with shedding of the head appendages, which becomes especially clear when the eyestalk is suddenly retracted from the eyecup of the exuvia (Fig. 3B,C). Active ecdysis is usually performed over 10–18 min, a time range similar to that of active crab ecdysis. Within the next few minutes, after the head appendages have been withdrawn, the carapace is lifted to an angle of approximately 90 ° to the longitudinal axis (Fig. 3C). This is then directly followed by abdominal shedding, which is characterised by peristaltic contractions of the abdominal segments (Figs 1, 3C–F; stages E_{50}–E_{100}). When abdominal shedding is approximately half completed, shedding of the pereiopods starts and is similar to the behaviour seen during crab ecdysis, with extensive contractions of the coxopodites and basipodites of the legs (Fig. 3D). Furthermore, these are accompanied by peristaltic vermiform movements of the meropodites within the exosketal tube of the old leg (particularly visible through a ‘window’ of decalcified cuticle in the meropodites of the first leg). Shortly after the onset of pereiopodal shedding, scaphognathite beating becomes visible through the transparent branchiostegites (Fig. 1; stage E_{75}). In contrast to the situation in the crab, abdominal withdrawal takes longer and lasts until the animal finally frees itself from the exuvia using two or three tailflips, which are initiated as soon as the last abdominal appendages, the uropods and the telson are withdrawn (Figs 1, 3F; stage E_{100}). The animals perform these final tailflips regardless of whether pereiopodal shedding is complete. This can consequently lead to the loss of appendages, most often of the chelipeds.

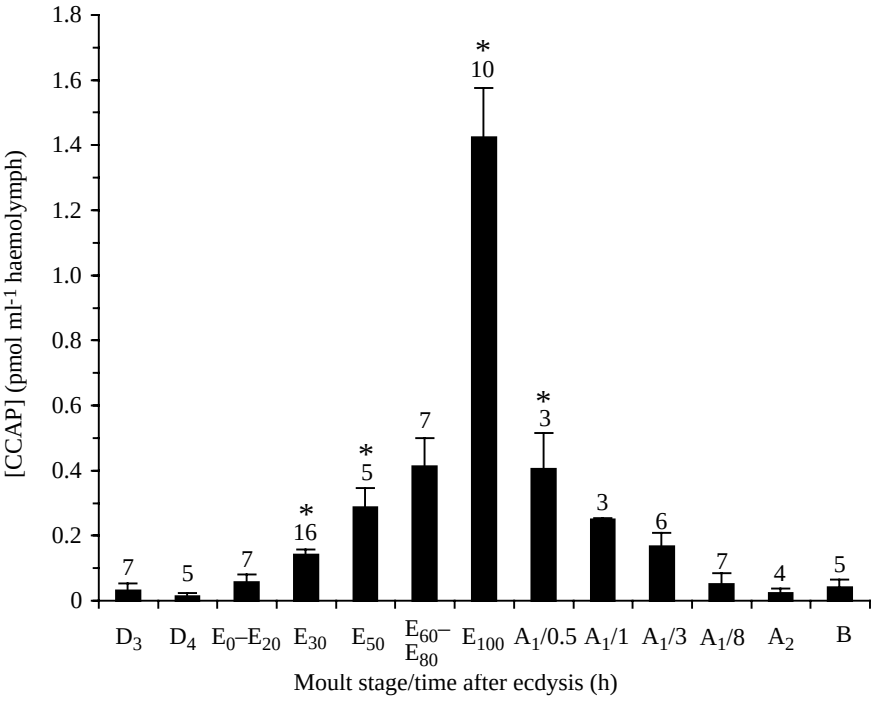

Fig. 6. Measurements of crustacean cardioactive peptide (CCAP) concentrations in haemolymph samples from shore crabs *Carcinus maenas* at different moult stages of adult individuals (carapace width 5–6 cm) of both sexes undergoing natural ecdysis. CCAP levels rise from the onset of active ecdysis (E_{30}–E_{50}) and reach a maximum of approximately 1.4 pmol ml^{-1} at the completion of the active ecdysis in stage E_{100}. Within 30 min after exuviation (A_1/0.5), the titre drops to less than 30 % of the E_{100} peak and reaches basal levels within the next few hours during A_1 examined at 0.5, 1.3 and 8 h intervals (A_1/1, A_1/3, A_1/8) after ecdysis. Values are means + S.E.M. and the numbers of specimens examined in each moult stage are indicated; asterisks indicate means significantly different from those of the preceding stage; P<0.0005 (Student's t-test).

Active ecdysis of the crab is associated with a large rise in CCAP haemolymph titre

The competitive EIA allowed the detection of CCAP (Fig. 4) at a limit below 5 fmol of CCAP per well (50 pmol l^{-1}), and the standard curve was routinely linear between 10 and 100 fmol of CCAP per well with an ED_{50} of 32 fmol of CCAP per well. Peptide recovery after Sep-Pak (C_{18}) purification was approximately 85 % (estimated using radiolabelled Y^2-CCAP), and samples of purified haemolymph showed curves parallel to those of authentic CCAP after logit/log transformations of dilution curves (Fig. 4, inset). Furthermore, the CCAP-immunoreactive fractions of purified samples co-eluted with the synthetic hormone after HPLC fractionation (Fig. 5A,B). This fact, combined with the parallel curves, indicated that the immunoreactive material was CCAP.

The first evidence of a large increase in the haemolymph titre of CCAP during ecdysis of the crab came from HPLC separations and subsequent assays of every fraction for CCAP immunoreactivity of intermoult (stage C_4) haemolymph in comparison with ecdysis haemolymph collected during active ecdysis at stages E_{80}–E_{100} (Fig. 5A,B). For both stages, 20 ml of pooled haemolymph was analysed, each collected from a group of 10 medium-sized crabs (5–6 cm carapace width) regardless of sex. The average intermoult titre of approximately 30 fmol CCAP ml^{-1} haemolymph (3×10^{-11} mol l^{-1}) in stage C_4 contrasted with a titre of at least 1000 fmol CCAP ml^{-1} haemolymph (10^{-9} mol l^{-1}) during active ecdysis (Fig. 5A,B). Similar haemolymph concentrations of CCAP were found during the active stage of ecdysis of destalked animals, which had been operated upon in early premoult (stage D_2).

The temporal pattern of CCAP haemolymph content of crabs in different premoult and postmoult stages and especially during the ecdysis substages was evaluated by analysing the CCAP titre in the haemolymph of individual animals (Fig. 6).

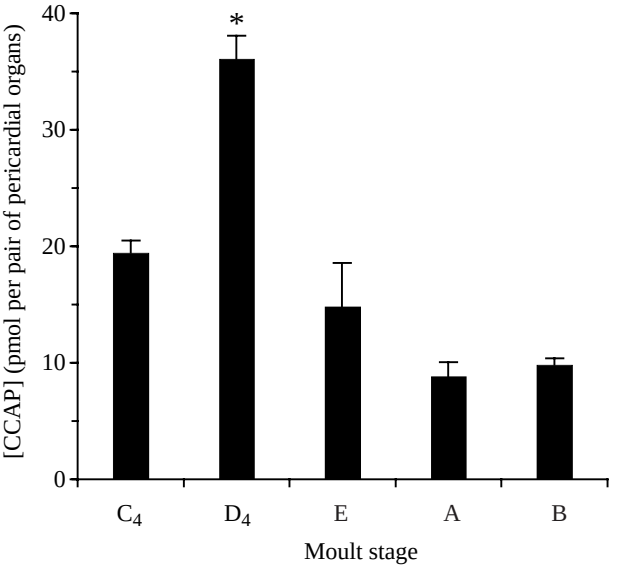

Fig. 7. Levels of crustacean cardioactive peptide (CCAP) immunoreactivity within the pericardial organs (extracted in 2 mol l^{-1} acetic acid) of shore crabs in different moult stages as determined by competitive enzyme immunoassay (EIA) (means + S.E.M.) showing that pericardial organs accumulate CCAP progressively in premoult stage D_4 to serve the massive ecdysial release of the hormone. The CCAP content of premoult pericardial organs is 2–3 times higher compared with the intermoult (C_4), ecdysis (E) and postmoult (A,B) stages; the asterisk indicates a mean significantly different from those of the adjacent stages C_4 and E; P<0.01 (Student's t-test).

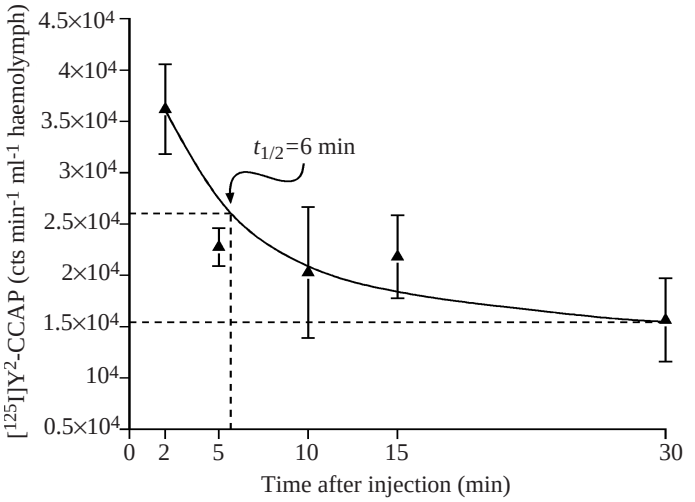

Fig. 8. Changes in haemolymph levels of a radioactive crustacean cardioactive peptide (CCAP) analogue after injection into shore crabs (5×10^5 cts min^{-1} of ^{125}I-labelled Y^2-CCAP injected). After subtraction of background radioactivity (1.5×10^4 cts min^{-1} ml^{-1}), an *in vivo* half-life ($t_{1/2}$) of [^{125}I]Y^2-CCAP of 6 min was calculated. Values are means ± S.E.M.

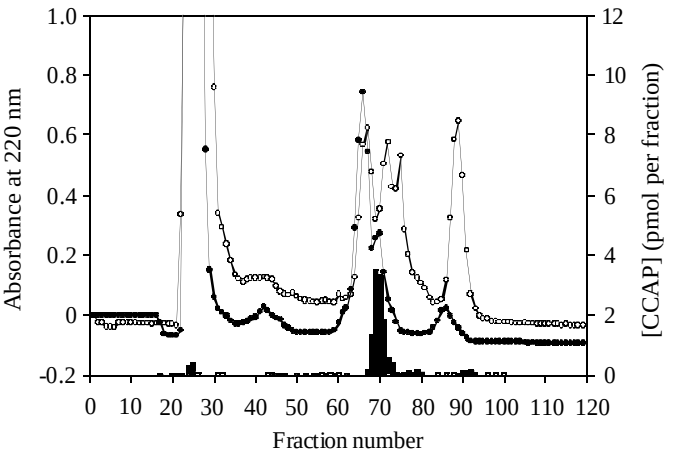

Fig. 9. Comparison of ecdysis (filled circles, filled columns; sampled during E_{100}) and intermoult (open circles, open columns) haemolymph (1 ml each) of two specimens of the crayfish *Orconectes limosus* by gel filtration and subsequent assay of each fraction for crustacean cardioactive peptide (CCAP) immunoreactivity by competitive enzyme immunoassay (EIA). The ecdysis animal shows a markedly high CCAP haemolymph titre of approximately 11 pmol ml^{-1} (fractions 68–72) compared with less than 100 fmol ml^{-1} in the haemolymph of the intermoult animal. Fraction size was 400 µl.

The timing of haemolymph sampling was based upon the morphological and behavioural criteria described above. With the onset of active ecdysis (stage E_{30}), the CCAP titre increased progressively and reached a maximum of approximately 1.4 pmol ml^{-1} haemolymph at the end of active ecdysis in stage E_{100}. Directly after ecdysis, the titres decreased to less than 30 % of the ecdysis maximum within 30 min (Fig. 6; stage A_1/0.5) and reached almost basal levels within the next 3 h during stage A_1 (Fig. 6; stage A_1/3).

Crab pericardial organs accumulate CCAP shortly before ecdysis

Pericardial organs were extracted from medium-sized male and female crabs (carapace width 5–6 cm) at different moult stages. The CCAP contents of these organs are 2–3 times higher in premoult stage D_4 than in intermoult (C_4), ecdysial (E_{80}–E_{100}) and postmoult (A,B) stages (Fig. 7). The CCAP contents in stage D_4 amounted to 36 pmol per pair of pericardial organs and, after ecdysial release, 15 pmol per pair of pericardial organs was found upon completion of stage E. Thus, one pair of pericardial organs releases more than 20 pmol of CCAP during the whole ecdysis process.

CCAP shows a remarkably short half-life in crab haemolymph in vivo

Injections into the circulatory system of intermoult crabs (N=2; stage C_4) of 5×10^5 cts min^{-1} of [^{125}I]Y^2-CCAP as a radiolabelled analogue of CCAP diluted in 100 µl of standard crab saline provided a first approach to estimating the half-life of CCAP in the haemolymph *in vivo*. Since it takes at least 2 min after injection until a substance is evenly distributed throughout the haemolymph (Webster, 1996), samples were taken 2, 5, 10, 15 and 30 min after the injection, during which time we observed an approximately exponential decline of sample radioactivity. At the end of the experiment, background radioactivity amounting to approximately 1.5×10^4 cts min^{-1} ml^{-1} remained in the haemolymph. After subtraction of this background, a half-life for [^{125}I]Y^2-CCAP of approximately 6 min was calculated (Fig. 8). Rapid dissection of the gills and the heart, immediately after taking the last haemolymph sample, showed that these organs bound less than 10 % of the injected peptide tracer (data not shown).

Crayfish ecdysis is also associated with a large increase in haemolymph CCAP titre

In experiments similar to those described for crabs, fractions obtained after gel filtration of haemolymph (1 ml per run) from single crayfish specimens in intermoult and ecdysis stages were analysed for CCAP content by EIA. In the haemolymph of an animal that had just finished ecdysis (see Fig. 3F; stage E_{100}), an enormous concentration of CCAP of almost 11 pmol ml^{-1} (1.1×10^{-8} mol l^{-1}) was found in contrast to less than 100 fmol ml^{-1} (10^{-10} mol l^{-1}) from an intermoult (stage C_4) animal (compare fractions 68–72 in Fig. 9). A minute amount of CCAP immunoreactivity was detected in the high-molecular-mass fraction of ecdysis haemolymph, which has not yet been analysed further (fractions 23, 24; Fig. 9).

Assays of haemolymph CCAP titres of 10 crayfish in different moult stages undergoing natural ecdysis (Fig. 10) resulted in an increase of more than 15-fold in CCAP content (range of maximum contents 5.5–11 pmol ml^{-1}) during active ecdysis (E) compared with the levels of premoult (D_4) or late

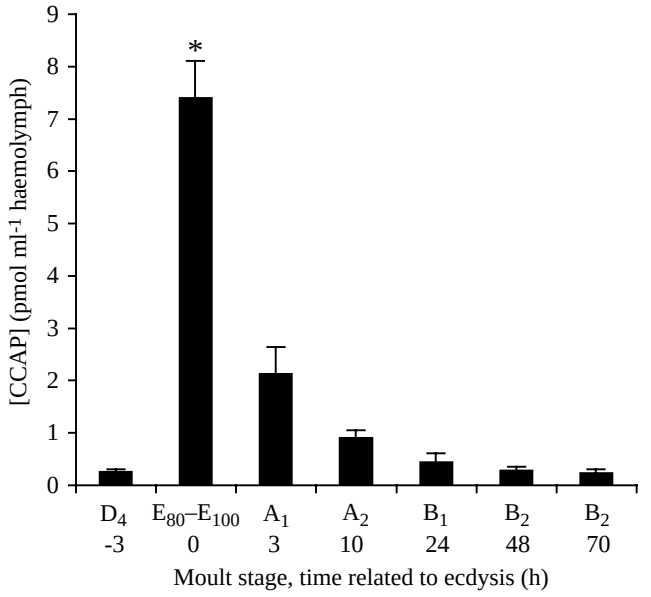

Fig. 10. Crustacean cardioactive peptide (CCAP) immunoreactivity in the haemolymph of the crayfish *Orconectes limosus* (*N*=10) at different moult stages and undergoing natural ecdysis showing that, during active ecdysis (samples taken in stages E_{80}–E_{100}), CCAP titres increase to 7.4 pmol ml^{-1}, which represents a more than 15-fold elevation compared with the levels in premoult (D_4) or late postmoult (A_2–B_2) stages. Note the rapid decrease in hormone titre within the first 3 h of ecdysis to approximately 30 % of the peak titres observed at ecdysis; values are means + S.E.M.; the asterisk indicates a mean significantly different from those of stages D_4 and A_1; P<0.00001 (Student's *t*-test).

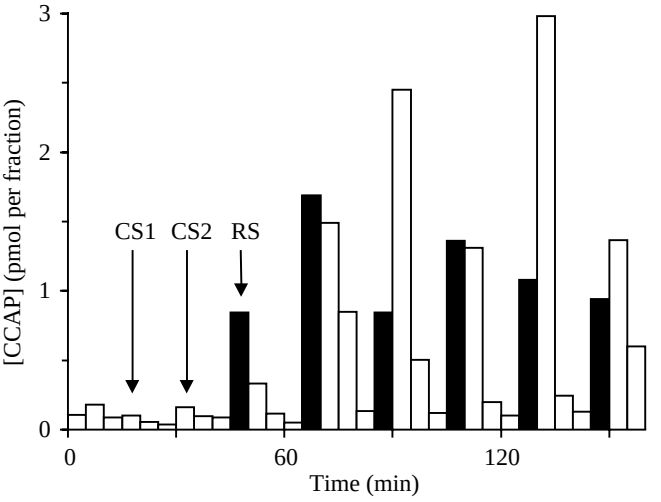

Fig. 11. Release of crustacean cardioactive peptide (CCAP) evoked *in vitro* from an isolated ventral nerve cord of the crayfish *Orconectes limosus* in intermoult (stage C_4) after six successive incubations with high-K^+ releasing saline (RS; 10×[K^+], 1×[Ca^{2+}], see Materials and methods; filled columns) followed by three steps in washing saline. Note that each RS incubation leads to the release of large amounts of CCAP into the washing saline to give approximately 3 pmol of CCAP in total and that the system is not exhausted even after six incubation cycles. Peptide release usually continues in the two following washing steps probably because of delayed exchange of diffusible ions within the ventral nerve cord after the saline change. Incubations with two different control salines (CS1, 10×[K^+], Ca^{2+}-free; CS2, 1×[K^+], 1×[Ca^{2+}]) caused no detectable release of peptide, indicating that the release is Ca^{2+}-dependent but does not occur spontaneously.

postmoult (A_2–B_2) stages. After ecdysis, the hormone titres decreased rapidly within the first 3 h to approximately 30 % of the peak value observed at active ecdysis and reached almost basal intermoult levels within the next 24 h (stage B_1). All samples of stage E were taken from animals during the last 5 min of active ecdysis (stages E_{80}–E_{100}; Fig. 6). Thus, the actual haemolymph CCAP levels at ecdysis are approximately five times higher in the crayfish than in the crab. Furthermore, a clear-cut increase in haemolymph CCAP concentration was detected in destalked animals during ecdysis. However, the maximum haemolymph titres in these animals reach approximately 2 pmol ml^{-1}, which is approximately five times lower than the titres found in the haemolymph of intact animals undergoing ecdysis.

Crayfish ventral nerve cords release large amounts of CCAP in vitro

Several *in vitro* experiments using release-inducing high-K^+ saline (releasing saline; RS) applied to isolated crayfish ventral nerve cords without brains showed that a large CCAP release can be evoked repeatedly. Whereas control salines (CS1, CS2) produced no significant release of CCAP above normal background levels (0.1–0.2 pmol), each incubation with releasing saline led to a release of approximately 3 pmol of CCAP. The system was not exhausted even after six successive incubation cycles (Fig. 11), since this large amount of peptide is only a small proportion of the content of a ventral nerve cord, which usually contains 70–80 pmol of CCAP. The hormone was typically detected in the releasing fraction and in two subsequent washing fractions, probably because of the relatively slow exchange of ions between the central parts of the ventral nervous system and the incubation saline. Notably, the use of washing saline or normal standard saline in the washing steps after high-K^+ stimulation (RS) led to essentially the same results. We have not been able to detect any striking difference between the *in vitro* CCAP release capabilities of intermoult (C_4) and premoult (D_4) ventral nerve cords with regard to the quantities of hormone found after incubation in releasing saline.

Discussion

The novel substaging using percentages of crab and crayfish ecdysis, designed following the criteria for a useful staging method suggested by Drach (1939), has allowed the substage-specific analysis of the temporal patterns of CCAP content in the haemolymph of two decapod species during natural ecdysis. This substaging system appears to be flexible, leaving space for further refinements in the future using details that we

may have overlooked or which may become apparent only after partial dissection of the integument of the animal, e.g. after removal of parts of branchiostegites or limbs. The massive increase in haemolymph CCAP concentration coinciding in both species with active ecdysis was followed by a rapid decline during the early postmoult stages to normal intermoult levels, which strongly suggests that the peptide participates in the regulation of active ecdysis.

Similar ecdysis behaviour in decapod crustaceans from different orders

Our approach of subdividing the passive and active phases of ecdysis in species from two different decapod crustacean orders, Brachyura and Astacura, has revealed remarkable similarities with regard to the behavioural elements required for successful ecdysis (Figs 1–3). This is largely in accordance with observations described earlier for several other decapod species of crab, crayfish and lobster (Vitzou, 1882; Drach, 1939; Passano, 1960; Aiken and Waddy, 1987; Waddy et al., 1995). Most aspects in earlier detailed descriptions, especially of the ecdysis of *Carcinus maenas* by Vitzou (1882), match exactly, although this author has overlooked the vital step of water uptake which, as has recently been established in this species, is probably driven by CHH released into the haemolymph from intestinal paraneurons (Chung et al., 1999). This water uptake leads to the visible opening of the crab carapace at predefined pleural sutures or to bulging of the intertergal membranes posterior to the crayfish cephalothorax, providing a definitive sign of the beginning of ecdysis, as previously noted by Réaumur (1712). Although the use of these signs still makes it somewhat difficult to assign a clear and distinct starting point for ecdysis, since water uptake by drinking (Neufeld and Cameron, 1994; Chung et al., 1999) is not an overt behaviour, we consider these signs to be unequivocal indicators for the beginning ecdysis. Interestingly, even after their thoracoabdominal membranes have ruptured (thereby providing access of environmental water to exuvial fluids in the apolysis space), neither species necessarily accelerates its ecdysis process; the animals may continue 'apparently normal' activities for an unpredictable period, especially under certain circumstances, e.g. if a potential predator is visible. Therefore, a 'point of no return' during crustacean ecdysis appears to be attained only later, i.e. directly after active ecdysis has started with its distinct overt wiggling behaviour followed by the remarkable horizontal movements of the antennules in the case of the crab, first noted in the present study, or the shedding of the eyestalks in the case of the crayfish. These behavioural elements definitively mark the onset of the stereotyped behaviour patterns that consist of a shedding sequence of head appendages, abdomen and pereiopods. The movements of the pereiopods, in particular, are characterised by alternating unilateral contractions, suggesting the existence of a basic motor program controlling decapod ecdysis, if not ecdysis of crustaceans in general. Once active exuviation is initiated, ecdysis is either completed or the animal dies.

Vitzou (1882) has highlighted some differences between brachyuran and astacuran crustaceans, namely the existence, in crabs, of an epimeral suture line preformed for rupture by water uptake. He further stressed that in astacurans, contrary to the situation in the brachyurans, the extrication of the head, cephalothorax and walking legs precedes that of the abdomen, as has been described in lobsters (Aiken, 1980; Waddy et al., 1995). However, in *Orconectes limosus*, we always observed that freeing of the abdomen occurs earlier or perhaps concomitantly with withdrawal of the chelipeds and other thoracic appendages. The same sequence has been reported for another crayfish (*O. virilis*), which died during these ecdysis steps, but does not form part of the naturally observed sequence (Aiken, 1968); thus, this sequence may be unique to the natural ecdysis of *O. limosus*. In fact, the final withdrawal of the chelipeds is always accompanied by brisk movements of the tail, an observation that also differs from that described for *O. virilis* (Aiken, 1968). These tailflips resemble those known to govern crayfish escape behaviour (Wine and Krasne, 1982). They may be initiated by sensory inputs from the uropods, because the crayfish tries to escape from its exuvia as soon as the last abdominal segment is withdrawn, regardless of whether shedding of the pereiopods is complete. Thus, for stereotyped ecdysis behaviour, preformed neural circuits are obviously recruited to serve the different and specialised behavioural repertoire for ecdysis. Pereiopod shedding by the crab is characterised by alternating (by side) and peristaltic contractions of the legs, in particular the coxopodites and basipodites, a movement that, according to Drach's earlier observations during ecdysis of the spider crab *Maja squinado*, resembles normal walking but may differ in that probably only the extensor muscles and not the flexor muscles of these leg compartments are being used (Drach, 1939). However, we do not favour the restrictive view of Drach (1939) that only these extensor muscles are involved in these movements, because we have regularly seen peristaltic-like muscle movements in other parts of the crayfish chelipeds during active ecdysis. Since in *C. maenas* the coxopodite contractions are performed regardless of whether the corresponding leg exists (e.g. after previous autotomy), it seems obvious that an underlying ecdysis motor program, once initiated, is independent of peripheral sensory inputs and probably cannot be interrupted. Furthermore, these alternating contractions correspond in terms of their temporal pattern to periodic changes in the hydrostatic pressure pulses, which are of much lower frequency than the heart rate during active ecdysis, as has been described for the blue crab *Callinectes sapidus* (deFur et al., 1985). These observations support the view that the haemolymph functions as a hydrostatic skeleton during ecdysis when the hard exoskeleton is shed and the new still-uncalcified skeleton is too weak to serve as a buttress or a stable tendon insertion point for proper muscle action (Drach, 1939; Mangum, 1992). Hardening of the shell in shore crabs during postmoult may take at least 3–16 days depending on the size of the crab, the temperature, the pH and the Ca^{2+} concentration in the water (Broekhuyzen, 1936).

Dynamics of CCAP release and its possible role during ecdysis of decapod crustaceans

The ecdysis of brachyuran and astacuran decapods is associated with a massive release of the neuropeptide CCAP into the haemolymph (Figs 6, 10). This event is restricted to the active ecdysis process, suggesting an important role for CCAP during complex ecdysis behaviour. Interestingly, while CHH titres in crab haemolymph show a similar temporal pattern during active ecdysis with a peak concentration at the end of ecdysis, CHH appears earlier in passive ecdysis, although at elevated but more moderate concentrations compared with premoult situations. During ecdysis, CHH certainly plays a role in the control of water uptake (Chung et al., 1999) and possibly also in energy resourcing during late active ecdysis (see below). The massive increase in CCAP titre beginning in E_{30} with a peak concentration at E_{100}, which drops during the first few hours of the postmoult phase (A_1), suggests that this peptide is necessary for the successful termination of the active part of ecdysis only. A remarkably similar temporal pattern of changes in CCAP content in the ventral nerve cord has been described previously in the isopod *Oniscus asellus* during ecdysis (Johnen et al., 1995). However, the increase in CCAP concentrations in late premoult stage D_4 suggested an accumulation of the peptide in the isopod ventral nerve cord prior to a presumed peak of release during posterior ecdysis. Levels of CCAP remain high after anterior ecdysis, approximately 1 day later. Peaks of immunoreactivity in CCAP neurons in the ventral nerve cord correspond to the posterior and anterior exuviation phases (H. Dircksen and C. Johnen, unpublished observations). Our observations concerning the transient change in the CCAP content of the crab pericardial organ extracts allow a similar interpretation, i.e. that CCAP is accumulated in the pericardial organs during stage D_4 to meet the high demands for release of the hormone during ecdysis.

Specimens of both species lacking eyestalks show the same transient elevation of CCAP titre during active ecdysis as intact animals, which suggests that the main source of humoral CCAP is probably the neurohaemal pericardial organs, as has been recognized previously in crabs (Dircksen and Keller, 1988; Stangier et al., 1988), or the ventral nerve cord (Trube et al., 1994) and the pericardial organs in crayfish, and is not under the control of classical eyestalk neuroendocrine systems. One pair of pericardial organs from an intermoult crab contains approximately 20 pmol of CCAP, which is in accord with earlier estimations (Stangier et al., 1988), and a single crayfish ventral nerve cord contains four times as much. Thus, the stored CCAP in the central nervous system and pericardial organs can entirely account for the extraordinarily high levels found in ecdysis haemolymph, even taking into account the rapid hormone turnover at this time, as shown by the estimations of CCAP half-life. The difference between the fivefold higher peak haemolymph concentrations of CCAP in crayfish compared with those in the crab is not understood at present. Moreover, whether there is a causative relationship between this accumulation of CCAP and the premoult peak of ecdysteroid titres will have to be a matter for future studies.

Ecdysis obviously demands a complex regulation of cardiovascular functions affecting heartbeat performance and the redirection of haemolymph flow, since prebranchial hydrostatic haemolymph pressures caused by the excessive water uptake are known to rise steeply (approximately fivefold) from stage D_4 onwards, reaching a maximum in stage E followed by a rapid decline in stages A_1 and A_2 (deFur et al., 1985; Mangum, 1992). It is known that CCAP is cardioacceleratory and slightly inotropic (Stangier and Keller, 1991; Wilkens, 1995) and elevates several variables of heartbeat performance in the crab such as cardiac output, stroke work, power output and intraventricular pulse pressure (Wilkens and McMahon, 1992; Wilkens and Mercier, 1993). It is also involved in the redirection of haemolymph flow into different regions of the body (McGaw et al., 1994; Wilkens et al., 1996) that are known to be associated with varying blood pressures during ecdysis (Drach, 1939; Passano, 1960; deFur et al., 1985). Such redirection of flow is a compensatory response to lowered oxygen contents of the blood and to abdominal movements in crayfish, lobsters and crabs, probably because the scaphognathite is less efficient during ecdysis (Kuramoto and Ebara, 1984; McMahon, 1992; Reiber et al., 1992). It is likely, therefore, that CCAP participates in the control of haemolymph pressure and flow.

Transient anoxic states during ecdysis, as have been demonstrated for exuviating blue crabs *C. sapidus* (Lewis and Haefner, 1976) and lobsters (Clemens et al., 1999), may be continued during a short postmoult period of anaerobic metabolism (Mangum et al., 1985b; Mangum, 1992). In blue crabs, this occurs concomitantly with a significant decrease in ventilation rate at ecdysis (deFur et al., 1985; Mangum et al., 1985a), although we have regularly seen overtly beating scaphognathites from stage E_{75} onwards. Since CCAP haemolymph titres are still moderately high shortly after ecdysis, the peptide could be involved in a stimulation of scaphognathite ventilatory activity, thus matching earlier observations at the end of ecdysis (Passano, 1960), and/or in pyloric motoneuron rhythmicity within the stomatogastric neural network, both of which are well-established effects of CCAP in intermoult animals (Stangier and Keller, 1991; Weimann et al., 1997). This view would be in accordance with events in blue crabs and lobsters shortly after ecdysis. For instance, Mangum et al. (1985a,b) observed increased haemolymph P_{O_2} and increased rates of O_2 uptake by several muscles of blue crabs during postmoult stage A_1, while lactate levels were still high. In lobsters, the lack of a relationship between persisting hypoxic conditions and the normal (compared with the slower ecdysial) pyloric network rhythmicity directly (approximately 10 min) after ecdysis has suggested that ‘unknown modulatory factors’ were influencing the neuronal activities at the end of ecdysis (Clemens et al., 1999). One of these factors may well be CCAP, which can both elicit and modulate motoneuronal activities in members of different arthropod groups (Mulloney et al., 1997; Weimann et al., 1997; Gammie and Truman, 1997a) and shows direct myotropic actions on several visceral muscle systems such as

those of the crustacean hindgut, the locust hindgut and oviduct and even the skeletal muscles of the metathoracic extensor tibiae in this insect (for reviews, see Dircksen, 1994, 1998). Consequently, a potentiation of muscular force at ecdysis may be considered a major function of humoral CCAP during and after the active crustacean ecdysis process, particularly since several limb muscles experience a reduction in mass of up to approximately 50 % during premoult compared with their original size and mass during intermoult (Mykles and Skinner, 1981; Mykles, 1997). A factor potentiating muscle power would thus be of great help, if not indispensable, during active ecdysis, which is probably the most power-demanding behaviour in the crustacean life cycle. Furthermore, this would call for the participation of CHH, which shows its highest titres at this time (Chung et al., 1999), in providing additional energy by mobilising glucose from glycogen stores.

Taken together, these results are indicative of a similar importance for the peptide CCAP during ecdysis in several malacostracan crustaceans and support the roles proposed earlier for CCAP as a highly conserved peptide in at least two putatively homologous CCAP neurons occurring in almost all different arthropod groups (Dircksen, 1998). Although, at present, we have no electrophysiological or immunocytochemical data on the behaviour of such CCAP neurons during ecdysis of crustaceans, their activity might be expected to resemble that of the well-documented CCAP-immunoreactive cell 27 (NS-L_1) and IN 704 groups in *Manduca sexta* (for a review, see Truman et al., 1998). These neurons exhibit large transient increases in immunoreactivity for CCAP and the second messenger cyclic GMP during the active parts of several ecdyses (Ewer et al., 1994, 1997; Ewer and Truman, 1996, 1997; Truman et al., 1996) and are the terminal executives of *Manduca sexta* ETH and EH action controlling the ecdysis motor programme in the insect ventral nerve cord (Gammie and Truman, 1997a,b, 1999). These neurons are the homologues of the type-1 and type-2 CCAP neurons that occur in several arthropod groups (Dircksen et al., 1991; Davis et al., 1993; Dircksen, 1994, 1998; Truman et al., 1998). The humoral release of CCAP, described here for the first time, is probably related only to the neurosecretory type-1 cells, which project into central and peripheral neurohaemal organs in crab and crayfish (Dircksen and Keller, 1988; Trube et al., 1994). Preliminary evidence from iontophoretic and backfilling studies (A. Trube, G. Plachta and H. Dircksen, unpublished results) suggests that these two neuron types are electrically coupled and thus must act simultaneously during arthropod ecdysis, thereby linking a central CCAP release onto motoneuronal networks executing the ecdysis motor programme to a parallel peripheral release that controls and modulates scaphognathite beating, systemic haemolymph pressure and flow alterations and probably several muscle systems. The humoral aspects seem to be in agreement with earlier reports on the involvement of cardioactive factors in the haemolymph of *M. sexta* during adult ecdysis and wing-spreading behaviour (Tublitz and Truman, 1985a,b), one of which is CCAP (Cheung et al., 1992) most likely released from the cell 27 group *via* perivisceral organs.

The discovery of an association between CCAP and crustacean ecdysis represents the first step in unravelling the most critical part (in terms of neuroendocrine and motor control) of the crustacean moult cycle, the ecdysis. Much has still to be learnt about the control of CCAP release and the putative target organs of this neurohormone at ecdysis. The lack of CCAP release in standard saline incubations (CS2; Fig. 11) in our crayfish ventral nerve cord *in vitro* system showed that there is no spontaneous release from CCAP neurons. Thus, the existence of some ecdysis-inducing factors might be expected, but there is no evidence for the existence of any *Manduca* ETH- or EH-like compounds in crustaceans. Our *in vitro* CCAP release assay in combination with the highly sensitive EIA provide a rationale for the elucidation of other neuroendocrine factors acting on CCAP neurons, thereby regulating the complex physiological and behavioural components of crustacean ecdysis.

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