# COST-FA1301 CephsInAction Survey
# Prospective Severity Assessment of procedures in Cephalopods

***Introduction***

Directive 2010/63/EU on the protection of animals used for scientific purposes, requires that a prospective assessment is made on the severity of each procedure in a Project (Article 15) and that a severity classification is assigned (i.e. "non-recovery", "mild", "moderate" or "severe"). Annex VIII of the Directive provides guidance on the factors to be taken into account in the assessment of prospective severity and gives some examples in each severity category, but these are based mainly on procedures in vertebrates.

As stated in the "National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes – Working document on a severity assessment framework", these measures provide opportunities to improve the quality of science and welfare through prospective review of project proposals and, by inclusion of the actual suffering experienced by the animal, should provide greater transparency and understanding of the impact of scientific procedures on animal welfare.

Specific guidance on severity assessment for cephalopods is lacking although is available for fish, based on a Norwegian Consensus-Platform for the 3Rs (norecopa) initiative (see Hawkins et al., 2011).

The primary aim of this COST Action FA1301 initiative is to derive an objectively-based prospective severity classification of procedures (within the meaning of Directive 2010/63/EU) for cephalopods which can be used to inform project applications to the National Competent Authorities.

**All members of COST Action FA1301 are invited to participate in the survey to facilitate the development of cephalopod-specific severity assessment criteria.**

The findings from the survey will contribute to enhancing the welfare of cephalopods in the laboratory and the application of the 3Rs. Information originated from this initiative will also help to to identify areas where we may need to provide resources to clarify issues (e.g. assessing "suffering" of a newly hatched cephalopod paralarva).

***The PAS-C Survey***

The Prospective Severity Assessment of procedures in Cephalopods survey comprises 50 mini-scenarios each giving a brief description of a procedure (see Guidance below) which you are asked to make an assessment about regarding:

a) whether it exceeds the threshold for regulation and;

b) if it does what the severity level is likely to be (prospective assessment) assuming it can be justified in any project application to the National Competent Authority.

The scenarios, although hypothetical are based upon procedures included in previously published works, including papers from more than 25 years ago; this does not necessarily means that they could be justified or would be authorized under Directive 2010/63/EU.

All the scenarios contain sufficient information to make an assessment, and the Guidance notes and Definitions of key technical terms provided here (derived from Directive Annex VIII) will help to identifying the main issues to consider in making the assessment.

Please read the Guidance notes before starting the Survey

You may also access to the Guidance notes while filling your responses

To identify the upper limits of the severity categories we have deliberately included examples of procedures which it may never be possible to justify, and inclusion should not be taken to indicate

that COST-FA1301 or anyone associated with it considers such procedures acceptable.

All responses will be treated confidentially and at no stage will you be identified by name. Information about individual Personal Profile is requested (see last pad of the Survey) since it is expected that the final study will compare various groups of respondents (e.g. senior vs junior researchers).

The results of the PAS-C survey will be presented at the November 2015 Annual Scientific meeting of the FA1301 COST Action in Lisbon (Portugal) and a summary of results will be posted on this website. A publication in a peer-reviewed journal is expected as final outcome.

***CephsInAction Prospective Assessment of Severity Survey is a milestone of WG4 and is coordinated by the FA1301 PAS subgroup***

Treat this survey as confidential and do not confer with others about your responses as we wish to know the independent views of as many participants as possible

***How the PAS-C Survey works***
To have access to the PAS-C survey you should be registered on the CephsInAction website and logged-in. As indicated, the responses you will give are confidential and there will be no connection between your login and the final result of the survey from each one of the responders.
At the PAS-C Survey page there are 10 pads (numbered 1 to 10) each containing five scenarios.
In each pad – corresponding to a block of five scenarios – it is always possible to look at Guidance and Definitions as an additional aid to facilitate the responses.
Each pad is independent and self-standing.
It is suggested to respond to all five scenarios included in each pad, before to proceed to the next one (button next at the end of each pad-page).
When logging-out, the information you provided will be automatically saved and retrieved the next time you will log-in.

Resuming your own activities on the survey will always ask you to start-over (i.e. start from the scratch, deleting all previously provided responses) or to continue keeping data you already provided. You can always revise and edit the responses you provided while logged-in

After completing the survey, please be sure that you have also provided information in the Personal Profile pad.

**This Survey will end in three weeks time. No late submissions will be allowed.**
No further editing and revision of the responses provided will be allowed after submission

***Guidance notes on how to assess the prospective severity of a procedure***

Notes and Definitions contained herein are derived from Directive 2010/63/EU (Annex VIII) and Severity Assessment Framework document.

The Directive emphasises that the severity assigned prospectively (i.e. the severity anticipated) to a particular procedure should be the "highest severity anticipated for any animal"; this emphasises the significance placed upon the experience of an individual animal rather than the "average" of a group.
A number of specific factors should be considered:
1. Nature of pain, suffering, distress or lasting harm (PSDLH) likely to be caused by (all elements of) the procedure, and its intensity, the duration, frequency and multiplicity of techniques involved.

2. Cumulative suffering within a procedure. This takes account of anything done to the animal within a procedure that may cause PSDLH; for example daily injection and stressful behavioural testing over a week in a procedure has a greater degree of potential cumulative suffering than if the animal was only exposed to these challenges once in a week.

3. Prevention from expressing natural behaviour including restrictions on the housing, husbandry and care standards (this includes food restriction and physical confinement).

4. Type of species and genotype (whenever applicable).

5. Maturity, gender and age (may not always be known for cephalopods) of the animal.

6. All species of cephalopod are covered by the Directive from the time of hatching (i.e. paralarvae and newly hatched cuttlefish are included).

For the purpose of this survey only, please assume the following general points about each scenario unless stated otherwise:
a. Catching. For all procedures you should assume that the animals were either caught from the wild by a competent person using the most humane method or were obtained from eggs either from the wild or from other captive animals.
b. Handling. Assume that all handling of animals is done by trained personnel using appropriate techniques
c. Acclimatisation to the laboratory. Unless indicated otherwise assume that in all cases that if an animal has been taken from the wild that the animal has become acclimatized to being housed in a laboratory aquarium before study.
d. General anaesthesia. Where general anaesthesia is mentioned assume that the most humane technique and appropriate anaesthetic agent has been used.
e. Post-operative recovery. Unless otherwise stated, where a surgical procedure has been performed you should assume that all possible measures have been taken to alleviate any post–operative pain.
f. Conduct of procedures. You should assume that all procedures (including ones involving surgery) are performed by appropriately qualified and trained personnel. Personnel will also be familiar with the any adverse effects of the procedure and predetermined humane end points.
g. Overall effects of the procedure. If the scenario mentions an effect of the procedure on the animal (e.g. reduced food intake) you should take this into account in your assessment. If no effect is

mentioned you should assume that the procedure has no effect that requires consideration in making your assessment.

# LIST OF SCENARIOS INCLUDED IN THE PAS-C SURVEY

## Scenario 1

Under general anaesthesia a small piece (3mm x 3mm) of the anterior fin is to be removed from juvenile (~50g) male cuttlefish (*Sepia officinalis*) without taking the animal out of the anaesthetic solution. Assume there are no postoperative analgesics available for use in cephalopods and that although evidence for wound directed behaviour in cephalopods is equivocal there is evidence for mechano-nociceptors and hypersensitivity following injury. The animal will be allowed to fully recover from the anaesthetic and there is no evidence that this lesion interferes with feeding or locomotion. Regrowth of the fin is to be followed by anaesthetising the animal for fin microphotography and ultrasound imaging every 5 days over the next 30 days to provide good temporal resolution of regrowth. The animals are expected to recover fully from each period of general anaesthesia which lasts <15min.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 2

In individually housed *Eledone cirrhosa* (adult males ~500g) behaviour is to be investigated. No aspect of normal care, welfare, feeding will be altered by the testing. Three tests are given in each session with the tests being carried out 10 minutes apart. There are 5 sessions (i.e. 15 tests) per day (total testing time is ~30min including 2x10min breaks) and the data collection lasts for 5 consecutive days for each octopus. *Alert test*: Observer opening the lid of the tank and recording the reaction of the octopus; *Threat test*: Octopus arm gently touched with a blunt plastic probe for 1s, all reactions recorded. *Feeding test*: Preferred live food provided in the opposite corner to where octopus was located, latency to attack and behaviours recorded.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 3

It is proposed that one hundred *Sepia officinalis* eggs are split into four groups with each group exposed to different live common cuttlefish predators. Each predator is housed with a group of eggs from laying until just before hatching. On hatching all the juvenile cuttlefish are shown visual images of each of the four predators with each presented in random order for 5 minutes with a 10min rest period between each exposure. The juvenile cuttlefish are provided with escape refuges and a substrate on which to camouflage while the reaction to the predators is recorded.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 4

Adult male squid (*Loligo pealei*) weighing ~100g will be removed from their home tank, held vertically and a semi-rigid narrow tube (2mm diameter) inserted into the crop via the beak. The animals will be given 1ml of an experimental drug via the tube. The tube is withdrawn and the animal returned to the water with the entire procedure lasting one minute. The drug has no known adverse effects on the animals. Groups of animals are to be killed at 30min intervals over the next 12 hours using a humane method and tissues removed to measure the drug distribution.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 5

It is proposed that in juvenile cuttlefish (*Sepia officinalis*, weight ~50g) under general anaesthesia the posterior sub-oesophageal mass is stimulated electrically using a microelectrode and the resulting skin chromatophore patterns mapped with the brain stimulation sites marked by dye injection. The animals will remain under general anaesthesia for the entire procedure and will be then killed by anaesthetic overdose prior to removal of the brain for histology.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 6

Adult male *Loligo pealei* (squid, weight ~100g) of similar size will be placed in a large tank and exercised on a single occasion using increased water current speeds. During the exercise period animals are under continuous observation and when they first show signs of fatigue the water current speed will be maintained until the animals become exhausted. Fatigue is indicated by an inability to maintain position in the water column and exhaustion by sinking to the bottom of the tank and not returning to the water column. The total swimming time to exhaustion is estimated to be ~45-60min. At the point of exhaustion animals are immediately humanely killed and tissues removed for analysis.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 7

It is proposed that incrementing concentrations of cuttlefish ink are added to tanks containing individual juvenile (~50g) cuttlefish (*Sepia officinalis*) until they show escape behaviours (e.g. inking, continuous jetting) or other signs of distress (e.g. deimatic display) to identify the threshold concentration. The animals will not be able to escape from the testing tank but as soon as they show escape behaviour/distress they will be returned to their home tank.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 8

Under general anaesthesia in adult (0.5kg-1.5kg) male *Octopus vulgaris* it is proposed to investigate the brain and arm morphology using non-invasive ultrasound over a 30min period with the animals immersed in a bowl of gassed sea water at home tank temperature and with ambient lighting subdued. Animals will be recovered and returned to their home tank after the procedure.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 9

It is proposed that adult male *Octopus vulgaris* (~450g) are removed from the water in their home tank and have the distal 1% of the tip of one arm (not R3) surgically removed. Within 2 minutes they will be returned to the water and left for 24 hours when they will be killed humanely and regenerating arm tissue removed for molecular studies. For this hypothetical example assume that no postoperative analgesics are available for use in cephalopods and also assume that a scientific justification is given in the project application that general anaesthesia may interfere with arm regeneration. Evidence that cephalopods exhibit wound directed guarding behaviour is equivocal but there is evidence for mechano-nociceptors in cephalopods and hypersensitivity following injury.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 10

To investigate potential analgesic agents (substances to reduce pain) it is proposed to use a mechanical stimulus to an arm and the dorsal mantle in adult *Octopus vulgaris*. The mechanical stimulus when applied at "high" (noxious) intensity is sufficient to induce the animal to move away from the stimulus and previous studies have shown that this stimulus intensity induced a learned aversion. At "low" (non-noxious) intensities the animals have a characteristic colour change and explore the area of the stimulus with an arm but do not withdraw and do not have a learned aversion to the stimulus. Using a Latin square design with a week between tests (see below) animals will be given either: a subcutaneous injection (0.1ml, 29G needle) of vehicle (sterile isotonic saline); an opiate with demonstrated analgesic properties in vertebrates and shown in in vitro studies to bind to cephalopod brain tissue; the opioid receptor antagonist naloxone which is hypothesised would block any endogenous analgesic pathways and would make the animals more sensitive to a noxious stimulus. Each test in the presence of either saline, opiate or naloxone consists of 5 challenges with the "low" intensity and 5 with the "high" intensity given in random order over a period of 50 min with the responses being recorded by high speed video. Assume that none of the drug treatments themselves have any adverse effects on the animals.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 11

Female *Nautilus pompilius* (~500g) will be deprived of food for 5 days (assume this is within the normal feeding interval range in the wild), and then fed on dead shrimps labelled with barium sulphate contrast medium (Note: this substance is used in clinically in humans for investigating digestive tract disorders by X-ray). Following dosing animals will be held in a large aquarium but every hour for the next 12 hours after feeding will be confined to the front of the tank for <5min where they will be exposed to X-rays to monitor the passage of the food (contrast medium). At the end of the procedure the animals will be killed by anaesthetic overdose, death confirmed and gut tissue removed for histology. (Note: at the dose used X-rays are not harmful to the animals).

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 12

The feeding preference for crab or shrimp is to be established in a group of adult wild caught cuttlefish (*Sepia officinalis*, ~700g) not habituated to the laboratory. Half of the animals will then be given their preferred prey coated with quinine (a bitter tastant in mammals) or the alternative prey. Up to 15 trials may be required for animals to stop attacking the prey coated in quinine. Assume that quinine has no adverse effects on the animal if treated food is ingested. Retention of a preference is tested by showing separate groups of animals live crabs or shrimp presented simultaneously one, two or three days after the preference has been established.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 13

It is proposed that juvenile (~ 50g) cuttlefish (*Sepia officinalis*) are placed in a small translucent box containing a substrate (sand/gravel) and small amount of seaweed within a larger tank. A known cuttlefish predator (Sea Bass) will be introduced to the large tank and all defensive signals made by the cuttlefish recorded over the next 60min. The cuttlefish will be able to hide from the predator using crypsis (substrate matching) or visually (amongst the seaweed).The predator will not be able to reach the cuttlefish and the water in the small (cuttlefish) and large (Sea Bass) tanks will not be in contact.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 14

Eggs from *Loligo vulgaris* are to be reared at simulated sea temperatures anticipated to occur as result of climate change. Based upon previous studies in other species it is expected that ~ 25% of hatchlings will have a malformation that impairs their ability to feed. All hatchlings (malformed and normal) are expected to survive for 4 weeks but the growth rate of the deformed hatchlings is expected to be reduced (~40% reduction). At 4 weeks all animals will be killed humanely and used for molecular analysis. What assessment would you give to the 25% of animals with a malformation?

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 15

Data logging tags are to be implanted into adult (~800g) cuttlefish (*Sepia officinalis*) in the laboratory to investigate their potential utility for studying animals in the wild. Under general anaesthesia the tag (~30mmx10mm, weight ~3g in air) is attached by superglue to the cuttlebone; this requires a small skin incision and drilling a hole 5mm wide and 5mm deep in the cuttlebone. After securing the tag the skin is closed around the wound edge using surgical sutures leaving the tag exposed on the surface of the animal. The wound edge is infiltrated with local anaesthetic prior to recovery from general anaesthesia. Animals are allowed to recover in individual tanks for 5 days before being placed in a large environmentally enriched communal tank for the next 14 days where there welfare is monitored twice daily. A previously published study indicated that within the time course of this study implanted tags have no overt adverse effects on health.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 16

It is proposed that adult ( ~800g) female *Octopus vulgaris* are placed individually in large (5m diameter and 3m deep) tanks with a vertical relief, mimicking natural conditions as far as possible and containing at least five potential dens. After a week an adult male (1.5Kg) will be introduced to each tank housing a female. All spawning/breeding behaviour is recorded, including agonistic interactions over a period of a week with multiple video-cameras. A previously published similar study reported that 10% of the female animals died and at autopsy showed fresh mantle and arm injuries consistent with being attacked. Note that the tank contains at least five potential den areas for refuge. What is your assessment for the female animals only?

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 17

Under general anaesthesia the mantle of adult (~500g) *Eledone cirrhosa* (octopus) will be partially everted (without surgery) and a 1.0ml sample of haemolymph removed from the branchial vein (25G needle; the estimated total haemolymph volume of the animal is ~30ml). The animal is allowed to recover and the above procedure repeated each week for 4 weeks.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 18

Two circular fiberglass tanks are to be used: a "small tank" and a "large tank" to investigate social interactions in cuttlefish (*Sepia officinalis*) Density in the small tank is 0.29 m2 per cuttlefish (i.e. higher than recommended density), whereas density in the large tank is 4.87 m2 per cuttlefish. Each tank will contain five adults (3F:2M) and have a sandy bottom, seaweed and some rocks but no refuges. Agonistic /courtship interactions will be observed with the study ending if/when potentially damaging behaviours are observed (e.g. jetting wildly, physical fighting. What is your assessment of the severity for the animals in the "small tank".

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 19

Adult (~1kg) *Octopus vulgaris* are to be studied in the following training regime. Twice daily while in their home tank they will be presented with either a black or a white plastic ball in pseudo- random order. Half of the animals will be rewarded with a shrimp if they take the white ball and if they take the black ball they will receive a brief electric shock (0.5sec, 10V, 50Hz) to the base of an arm sufficient to cause withdrawal of the animal. Based on previous studies some animals are expected to learn to discriminate the balls after 5 days but others are likely to take up to 10days (i.e. an individual may receive electric shocks daily for up to 10 days). When animals have learned to discriminate they will be killed humanely and their brains removed for analysis.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 20

The growth rate and behaviour of groups of *Sepia officinalis* from hatching to one month old will be compared using their normal diet and three artificial diets which will also fulfil the nutritional needs of the animals. The tanks will be under continuous video recording from the side and above for behavioural analysis. Each week over the month of the study under general anaesthesia the animals will be weighed, external measurements taken and the length/density of the cuttlebone measured using ultrasound. Assume that short periods of general anaesthesia have no adverse effects on the health of the animal over the month and that they recover rapidly form each exposure. At the end of the month the animals will be killed by a humane approved method and body composition analysed.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 21

It is proposed that incrementing concentrations of cuttlefish ink are added to tanks containing individual juvenile cuttlefish (*Sepia officinalis*, ~200g) until the juvenile cuttlefish shows escape behaviours (e.g. inking, continuous jetting) or other signs of distress (e.g. deimantic display). The animals will not be able to escape from the testing tank but as soon as they show escape behaviour/distress they will returned to their home tank. One week later 6 animals will receive either an intramuscular injection (0.1ml, into the side of the neck where there is a high blood flow using a 29G needle) of a drug with possible anxiolytic properties (assume it has no adverse effects on the animals and that the dose used is based upon published studies in a comparable species) or drug vehicle (0.1ml, isotonic sodium chloride) and are then exposed to the concentration of ink previously shown to induce escape behaviours and their reaction assessed over an hour. They are then returned to their home tank for a week and the above challenge repeated with animals receiving drug now receiving vehicle and vice versa. Animals will then be humanely killed.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 22

Newly hatched paralarvae of *Octopus vulgaris* (<1mm, DML) will be killed by immersion in liquid nitrogen for subsequent biochemical analysis.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 23

As part of a study to investigate factors (e.g. optic gland secretions) regulating post-reproductive life span the following study is proposed as one of the "controls" for the larger study. It is proposed that six mature female *Octopus hummelicicki* (~300g) bearing fertilised eggs will be kept in the laboratory until the eggs are laid after which they will care for the eggs. Following removal of the paralarvae for another project the adult females will be transferred to a tank (design compliant with current guidelines) equipped to monitor their oxygen consumption, ammonia production and overall locomotor activity all using non-invasive methods. Animals will be offered food daily and remain in the monitoring tank until they die of natural causes. The literature indicates that post -reproduction animals live for about 6 weeks and that they have a reduced/absent appetite, lose weight and may develop cataracts and skin lesions which may become infected. No treatment is available for the skin lesions or for any infections but if any animals lose 20% of their body weight, exhibit cataracts or skin lesions veterinary advice will be sought immediately and a decision made whether the affected animal should be killed humanely.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 24

Adult male *Octopus vulgaris* (~1Kg) will be anaesthetised the distal 10% of one arm (not R3) will be surgically removed. As no postoperative systemic analgesic agents are available for use in cephalopods the wound area will be infiltrated with local anaesthetic that is expected to produce a nerve block for 1-2h. Within 2 minutes of surgery they will be returned to the water and left for 24 hours when they will be killed humanely and regenerating arm tissue removed for molecular studies. Evidence for wound directed guarding behaviour is equivocal in cephalopods although there is evidence for the existence of mechano-nociceptors and hypersensitivity following injury.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 25

It is proposed that healthy adult *Octopus vulgaris* (~1Kg) will be fed for 5 days on live crabs inoculated with a newly identified parasite following a pilot study showing this protocol reliably caused infestation. At the end of the 5 days the following parameters will be measured and then again every 10 days for the next 3 months: body weight (involving removal from the water for <60s); testing of behaviour using an established behavioural battery known to not induce pain, suffering, distress or lasting harm; sampling of blood (from the branchial heart) under light general anaesthesia for 5min. From previous observations in the wild the parasitic infection is known to produce skin lesions covering a maximum of 5% of the body in about 50% of the animals after about two months. To enable a comparison of animals with and without skin lesions it is proposed that all animals will be kept for the entire three months before being killed humanely. No treatment is available for the lesions.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 26

Ten day old *Sepia officinalis* (<5g body weight) will be killed by direct immersion in fixative (formalin).

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 27

*Watasenia scintillans* (firefly squid) eggs will be allowed to hatch and then split into groups of 50 with each group receiving one of 5 different experimental paralarvae feeds and compared with a feed ("standard diet") shown in a previous study to provide adequate growth and to have no overt adverse effects. Although the composition of each of the 5 experimental feeds is based upon the analysis of the natural diet the palatability for the animals is not known. It is also not know whether if the animals ingest the diet that essential nutrients will be absorbed equally well in each formulation. Each day over the next month the survivors are counted to assess the efficacy of the diets. If the daily mortality exceeds 20% for any diet that diet will be withdrawn and remaining animals placed on the "standard diet" for the remainder of the month of the study.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 28

It is proposed to use a method to kill adult squid (*Loligo vulgaris*; ~1Kg) that is not described in the Directive (Annexe 4) nor recommended in Guidelines for the Care and Welfare of Cephalopods; therefore the proposed method is described in detail in a project application with a justification. It is proposed that animals will be removed from the water and decapitated (transection between the head and mantle) by a guillotine within 30 sec by a trained technician. The brain is then removed for preparation of an *in vitro* brain slice for neurophysiological studies.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 29

In adult (~750g) *Eledone cirrhosa* it is proposed that under general anaesthesia which is maintained for the whole experiment the left stellate ganglion will be surgically exposed to enable electrical stimulation of small groups of neurones using a microelectrode and the dorsal mantle will be monitored by high resolution video recording. A catheter will be placed in the systemic heart to allow administration of drugs to investigate their effect on the responses to stellate ganglion neurone stimulation. At the end of the experiment the animal is to be killed by anaesthetic overdose and tissues including the brain harvested for other studies.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 30

Under general anaesthesia it is proposed that all the supraoesophageal lobes of the brain will be removed from adult (~1500g) male *Octopus vulgaris* leaving the suboesophageal lobes intact. Post-operative analgesia is provided for 48h after surgery. This procedure has been equated to "decerebration" in vertebrates (see Notes below). Animals are allowed to recover from the anaesthesia and surgery (one week) and over the following two weeks are used to investigate the ability of the animals to respond to a noxious stimulus (a single electric shock to the mid-point of one arm) applied daily.
**Note:** assume that the animals are still able to feed and maintain normal homeostasis. In mammals and other vertebrates "decerebration" is a procedure performed under general anaesthesia during which all parts of the brain rostral to the colliculi are removed (i.e. the entire cerebral hemispheres) leaving the pons and the brainstem. The animals recover from the anaesthesia and with appropriate care can live for extended periods of time. The procedure removes all parts of the brain implicated in perception and consciousness. In *Octopus vulgaris* surgical removal under general anaesthesia of all the supraoesophageal lobes (including the vertical and frontal lobes) is considered to be the equivalent of decerebration in a vertebrate. The suboesophgaeal lobes will continue to function and maintain basic physiological functions but all the "higher lobes" implicated in conscious perception of external events and hypothesised to be involved in higher level processing of signals from nociceptors are removed.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 31

It is proposed that newly hatched paralarvae of *Octopus vulgaris* are sedated by exposure to sea water that is progressively cooled from ambient to near freezing. When movement and breathing stops the sea water is rapidly replaced by a sea water-formalin solution at near freezing temperature to fix them for later study.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 32

In their home tank *Octopus vulgaris* (500-700g) will be deprived of food for 24hours (there is no evidence that this period of food deprivation has any adverse effects), fed mussels labelled with an inert marker and then the animals will be observed continuously for production of faecal ropes which will be collected and analysed for the presence of the inert marker and corticosterone. The animals will not be used subsequently in any regulated procedures.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 33

It is proposed to expose sub-adult squid (*Loligo vulgaris*, ~200g) to low frequency sound in their home tank for a period of two hours. They will then be killed by a humane method for analysis of their statocysts. Previous studies in cephalopods indicate that the intensity and duration of the sound exposure to be used would lead to permanent damage to the statocysts and impair the ability of the animal to maintain its position in the water column. No other adverse health effects are known to occur within the two hour time period of this study.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 34

It is proposed that a small transparent tank containing a live prawn is placed in a tank containing an adult cuttlefish (*Sepia officinalis*, ~750g). The cuttlefish is able to see and attack the prawn but can't access it. Time to inhibition of predation/attack response is recorded. The cuttlefish are then to be retested the next day, and again for 5 consecutive days. All cuttlefish are fed their normal frozen laboratory diet 3 hours after each test. For testing individual cuttlefish will be trained to enter a smaller testing tank in direct continuity with the larger communal tank where they are normally housed.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 35

It is proposed that adult male *Octopus vulgaris* (~450g) are removed from the water in their home tank and under general anaesthesia the distal 10% of the tip of one arm (not R3) surgically removed. Within 2 minutes they will be returned to the water and left for 24 hours when they will be killed humanely and regenerating arm tissue removed for molecular studies. For this hypothetical example assume that no postoperative analgesics. Evidence that cephalopods exhibit wound directed guarding behaviour is equivocal but there is evidence for mechano-nociceptors in cephalopods and hypersensitivity following injury.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 36

It is proposed that a group of adult female *Sepia officinalis* (~600g) will be divided into two to examine the effect of food deprivation on body biochemistry. One group will be fed daily with a normal diet meeting all nutritional needs (control) and the second group will be deprived of food for 7 days. At the end of 7 days all animals are to be killed by a humane method and tissue samples removed for molecular studies. What severity would you grade the animals in the food deprived group. Assume that in the wild a cuttlefish would normally be expected to feed no less frequently than every 2 days and that in the laboratory they are likely to have been fed daily although food is not available ad *libitum*

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 37

It is proposed that twice daily adult *Octopus vulgaris* (~ 600g) are presented with a black or white plastic ball in pseudo- random order. Animals are rewarded with a shrimp if they take the white ball and if they take the black ball they receive a brief electric shock (0.5 sec, 12V, 50Hz) to the base of an arm sufficient to cause withdrawal of the animal. In each group (shrimp/shock) half of the animals will receive a daily intramuscular injection (100µl, 29G needle) of either a drug intended to affect memory consolidation or vehicle (isotonic sodium chloride) into the base of an arm. Different arms are injected each day of the 7 days of the study. Neither the drug nor vehicle are expected to have any obvious adverse effect on the animals. At the end of the 7 days animals are killed humanely and brains removed for analysis.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 38

*Octopus vulgaris* (males, ~500g) caught from the wild (using traps) in two locations with a different bottom (mainly rock vs mainly sandy) will be housed individually in tanks (compliant with guidelines) containing a terracotta pot, some rocks and shells. The animals are to be placed in these tanks immediately on arrival in the research facility aquarium. All environmental parameters are within guideline recommendations. The animals are fed daily on crabs previously established as the preferred food for animals from these locations. The animals will be under continuous video recording for a month to investigate if there is a difference in the pattern of arm use between animals from the two locations. At the end of the month animals are returned to the place of their capture following certification by the veterinarian.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 39

It is proposed to study the process of regeneration in *Abdopus aculeatus* (~1Kg) in which an arm has been induced to autotomize by a crush injury of the proximal part of an arm using forceps applied for up to 20s. It is not proposed to use either general or local anaesthesia as it is argued that these may interfere with autotomy (for this scenario only assume that this argument is accepted) and analgesics are not available for use in cephalopods. Following autotomy groups of animals will be killed humanely at intervals between 1 and 7 days and tissues harvested for molecular studies.
**Note:** autotomy is a natural occurrence in this species in the wild. The evidence that cephalopods "guard" injured parts of the body is inconsistent. There is evidence that octopus has mechano-nociceptors and that general cutaneous/muscular hypersensitivity persisting for the duration of this procedure may occur. Autotomy of one arm will not interfere with the ability of the animal to feed or groom.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 40

It is intended to compare the growth rate and behaviour of *Sepia officinalis* from hatching to one month using their normal diet and three artificial diets which will also fulfil the nutritional needs of the animals. The tanks will be under continuous video recording from the side and above for behavioural analysis and each week frames will be taken from the videos to measure the size of the animals. At the end of the month the animals will be killed by a humane method and body composition analysed.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 41

It is proposed to use a method to kill adult octopus (*Octopus vulgaris*; up to 1.5Kg) that is not described in the Directive (Annexe 4). Animals will be removed from the water and the brain irreversibly physically damaged using a small version of the captive bolt gun permitted for killing some vertebrates. The entire procedure will take <30sec for the time of removal from the water.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 42

The food preference of captive *Argonauta argo* (Paper Nautilus; ~200g) is to be investigated by presenting different foods to the arms. The foods are a small piece of fish, shrimp, crab or a cube of an artificial diet used to feed cuttlefish. One food is to be presented each day and the responses to food presentation will video recorded. The study will end when each food has been presented to the animal on 10 occasions. There are no alterations to any aspect of normal animal care as part of this study.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 43

It is proposed to investigate the pathophysiology of a novel potential environmental pollutant by exposing groups (10 per group; 10groups) of adult female cuttlefish ( ~500g ) to increasing concentrations) of the suspected chemical. The study aims to identify the $LD_{50}$ (the concentration causing death in 50% of the animals) with all dead animals being subject to autopsy. Incidental observations of cuttlefish in the wild indicate that death occurs 5-15 days from first symptoms which include decreased feeding, skin lesions (particularly ventral skin) which fail to heal and frequently become infected, cataracts and loss of buoyancy resulting in uncoordinated swimming. Assume that symptoms occur in all animals and increase in severity and number until death occurs.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 44

Adult blue ringed octopus (*Hapalochlaena lunulata*; weight <50g) will be anaesthetised and the left mantle connective (containing the nerves connecting the suboesophageal lobes to the left stellate ganglion) crushed using forceps. On recovery from the anaesthetic the left side of the mantle muscle will no longer contract and there will also be a loss of chromatophore activity and the ability of the skin to form papillae on the left side. There are no other obvious effects of the lesion on the health of the animal. Over the next three months the regeneration of the mantle connective nerves will be followed by monitoring recovery of mantle and skin function using high resolution video recording of the animal once a week in a small chamber (10cm3) located in its home tank which it had previously been trained to enter using a food reward. Each video recording session will last one hour. At the end of three months the animals will be killed humanely and tissues removed for analysis.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 45

The genome of *Grimpoteuthis sp.* (Dumbo octopus) is to be mapped by taking 2mm of tissue from the tip of arm L3 using a sterile razor blade with the animal briefly removed from its home tank (<30sec). It is proposed to do this without anaesthetising the animal as the risk of death from anaesthesia is considered to be high and these are relatively rare animals, difficult to keep in captivity. Following recovery and certification by a veterinarian the animals will be placed in a specifically designed public display aquarium.

- ( ) Subthreshold
- ( ) Non-recovery
- ( ) Mild
- ( ) Moderate
- ( ) Severe
- ( ) Upper threshold
- ( ) Unable to decide

## Scenario 46

Under general anaesthesia it is proposed that adult cuttlefish (*Sepia officinalis*) will have the left optic lobe surgically exposed. Under continuing general anaesthesia recordings of neural activity will be made from the optic lobe to investigate how the optic system processes polarised light. The animals will remain under general anaesthesia for the whole experiment with cardiovascular and respiratory parameters maintained within normal limits. The experiment will last ~6hours and at the end the brain will be perfused with formalin via the dorsal aorta and removed for histological analysis of recording sites which were marked with dye during the experiment.

- ◯ Subthreshold
- ◯ Non-recovery
- ◯ Mild
- ◯ Moderate
- ◯ Severe
- ◯ Upper threshold
- ◯ Unable to decide

## Scenario 47

It is proposed that adult male *Octopus vulgaris* (~500g) will be removed from the water in their home tank. A sterile needle (25G) will be inserted into the mid-point of one arm and local anaesthetic injected to block transmission in the nerve cord; this effect will last for about 2 hours. The animal will be kept for 15 mins in a small tank while the local anaesthetic takes effect. The animal is then to be removed from the tank and the distal 50% of one arm (not R3) surgically removed distal to the nerve block but the cut end is not surgically repaired so that the wound mimics injuries in the wild. Within one minute the octopuses will be returned to the water and left for 24 hours when they will be killed humanely and regenerating arm tissue removed for molecular studies. For this hypothetical scenario assume that no analgesics are available for use post-operatively in cephalopods and that evidence for wound directed guarding behaviour is equivocal in cephalopods although there is evidence for the existence of mechano-nociceptors and hypersensitivity following injury.

- ◯ Subthreshold
- ◯ Non-recovery
- ◯ Mild
- ◯ Moderate
- ◯ Severe
- ◯ Upper threshold
- ◯ Unable to decide

## Scenario 48

It is proposed to investigate whether a particular chemical has general anaesthetic properties in male *Octopus vulgaris* (~400g). Animals will be habituated to enter a small tank measuring 30cm3 containing gassed sea water at the same temperature as the home tank. This chamber will be used for the anaesthetic investigations. The aim is to identify a concentration of the agent which will induce general anaesthesia and from which the animal will fully recover. The potential agent will be delivered in an increasing concentration in sea water to the final desired concentration over a period of 15min. If the animal shows an aversive response (e.g. inking, increased rate of breathing) it will be returned to sea water. If the animal does not react and does not show signs of anaesthesia after the 15min exposure to the substance the concentration will be doubled and the response monitored for a further 15min. This process of escalating the concentration will continue until either the animal's shows signs of general anaesthesia or until a concentration 8x that of the original concentration is reached. Assuming that an anaesthetic concentration is reached the animal will remain in that concentration for 15min whilst it is tested to see if it fulfils criteria for general anaesthesia including the response to a noxious stimulus applied to an arm and the mantle. Other criteria will be skin pallor and loss of the righting reflex. Cardiovascular function and mantle movement will be assessed using non-invasive ultrasound. Following assessment of anaesthesia the anaesthetic solution will be replaced with fresh sea water and recovery monitored. When the animal is fully recovered it will be returned to its home tank and monitored hourly for the next 6 hours during which time it will be offered food.

- ◯ Subthreshold
- ◯ Non-recovery
- ◯ Mild
- ◯ Moderate
- ◯ Severe
- ◯ Upper threshold
- ◯ Unable to decide

## Scenario 49

It is proposed that juvenile cuttlefish (*Sepia officinalis*, ~50g) are placed in a small transparent box containing no refuge or materials within which to hide. The small transparent box has holes (1mm diameter) that allows free exchange of water with the larger tank in which the small box is located. A known cuttlefish predator (Sea Bass) is introduced into the large tank and the behaviour of the cuttlefish in the small tank will be recorded for 60min after which the predator will be removed. During the test the juvenile cuttlefish will not be able to hide from the predator but the predator will be unable to reach the cuttlefish.

- ◯ Subthreshold
- ◯ Non-recovery
- ◯ Mild
- ◯ Moderate
- ◯ Severe
- ◯ Upper threshold
- ◯ Unable to decide

## Scenario 50

Newly hatched paralarvae obtained from captive adult female *Octopus vulgaris* will be exposed for 24hours in groups to a range of different concentrations of ammonia or nitrite in oxygenated sea water. At the end of 24 hours the number of animals swimming or dead will be measured and used to calculate the median lethal concentration ($LC_{50}$).

- ◯ Subthreshold
- ◯ Non-recovery
- ◯ Mild
- ◯ Moderate
- ◯ Severe
- ◯ Upper threshold
- ◯ Unable to decide

**CephsInAction**
Thank you for your time and cooperation