APPROVED PROTOCOLS FOR DECAPODS, CEPHALOPODS AND FISH

Animal Ethics Sub-Committee
of the
University of KwaZulu-Natal
Ethics Committee

TABLE OF CONTENTS

1 STANDARD PROTOCOLS FOR DECAPODS (CRAYFISH, CRABS AND TRUE SHRIMPS AND PRAWNS)

1.1 OVERVIEW

Decapoda is one of the orders in the class Malacostrata of the subphylum Crustacea (phylum Arthopoda). Decapods are therefore not vertebrates, but they employ discrimination learning to avoid pain (Magee and Elwood, 2013), and ethical approval is needed to conduct research on decapods at UKZN.

The order Decapoda ("ten-footed" crustaceans) include crayfish, crabs and true shrimps and prawns. Although popular research models such as isopods, amphipods and mysids fall under the class Malacostraca, they do not belong to the order Decapoda, but the orders Isopoda, Amphipoda and Misida respectively. Brine shrimp (*Artemia*), and *Daphnia* are also not decapods, and belong to a different class altogether (class Branchiopoda). Please see the table below for detail (only orders of the class Malacostraca are highlighted here):

SUBPHYLUM	CLASS	ORDER
Crustacea	Branchiopoda (e.g. *Artemia* (brine shrimp); *Daphnia*)	
	Remipedia	
	Cephalocarida	
	Maxillopoda (e.g. copepods)	
	Ostracoda	
	Malacostraca	**Decapoda (e.g. crayfish, crabs, true shrimp & prawns)** Isopoda (isopods) Amphipoda (amphipods) Stomopoda Mysida (mysids) + 12 others

1.1.1 Useful guideline documents

This guideline is not exhaustive and all methods/procedures should be suitable for the specific species/experimental conditions/endpoints measured and must be supported by evidence from literature. Some detailed methods/procedures can be found in:

Canadian Council on Animal Care Guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing. http://www.ccac.ca/Documents/Standards/Guidelines/Appropriate_endpoint.pdf

Canadian Council on Animal Care Guidelines on: Euthanasia of animals used in science.

http://www.ccac.ca/Documents/Standards/Guidelines/Euthanasia.pdf

All research involving collecting decapods are subject to international, national and provincial legislation and policies covering collection and transport of animals (please see section 10 in the *AREC Guide to the Care and Use of Animals in Research and Teaching*). The necessary permits and permission need to be obtain prior to the start of the project as per AREC guidelines, including State Veterinary certificates for movement of animals where needed. The local fisheries/conservation officers must at all times be notified prior to collection of wild decapods.

1.1.2 Selection of Experimental Procedures/Endpoints

When using live animals in research one must aim to select endpoints appropriate for the outcomes of the experiment, whilst minimising pain and distress to the animal. In addition, endpoints indicating discomfort, pain and morbidity should also be defined and humane endpoints for euthanasia should be pre-determined. The *Canadian Council on Animal Care Guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing* provides detailed guidelines.

Some specific points:

- Animals should be carefully monitored during acclimation and experimentation and a table similar to that in **14.16** can be used to record animal health parameters. All observations should be conducted by trained personnel.

- A small pilot study might be required to test whether the endpoints, number of animals (via power analysis) and observation frequency selected are suitable for the experiment. This also allows for training of personnel.

- While the Organisation for Economic Cooperation and Development (OECD) discourages acute toxicology studies with death as an endpoint (i.e. standard lethal toxicity testing), well-motivated studies will be considered, if the OECD protocols (www.oecd.org) are closely followed to minimise animal numbers and all possible care is taken to relieve animal discomfort, pain and distress.

- Studies involving the exposure of decapods to environmental extremes should select the earliest endpoint possible.

The least stressful procedures should at all times be selected and all procedures must aim to inflict minimum harm, morbidity and mortality. All procedures must only be carried out by competent individuals. All para-veterinarian procedures must be carried out under supervision/in consultation of the University veterinarian. Veterinary procedures may only be carried out by a registered veterinarian. South African Veterinary Council (SAVC) certification/authorisation may be a requirement for certain procedures and it

is best to consult with Animal Care technicians in in the Biomedical Resources Unit/the resident veterinarian during the planning phase of the project.

1.2 CAPTURE (Code: DC)

There are multiple methods for collection of crabs, including hand collecting, netting, baiting and trapping. The collection method should match the species collected and the environment from which animals are collected (e.g. ocean/estuary/river/dam). These methods should be adequately outlined and supported by literature. It is important to note possible effects of the collection method on the animal's health, or that of non-target taxa. Netting may damage eyestalks and appendages. Nets with an appropriate mesh size should be used at all times, and excessive weight loading at the bottom of nets should be avoided. The bursa of female crabs should be checked for young, and collection during moulting should be avoided. The animals are particularly prone to succumb to stress during their moult if kept in artificial environments.

1.3 TRANSPORT (Code: DT)

Decapods should be transported only once necessary permits/veterinary certificates are in place. Transport conditions should inflict as little stress as possible. The container should be of the appropriate size, well insulated to prevent changes in temperature and light intensity, and should not leak. The container should be disinfected before use, and water quality must match the appropriate water quality standards. Care should also be taken to match water quality between the water body where animals are collected from, the transport container, and the tank in which animals will be kept in the laboratory to avoid osmotic/thermal shock. Because decapods are ectotherms, it might be appropriate to use slightly cooler water during transport to lower metabolism. If possible, decapods should be fasted for 12-48 hours prior to transport and this time will depend on species, age and water quality. This minimizes the contribution of faeces to ammonia loading of the water. Although decapods are generally ammonia tolerant, ammonia-nitrogen levels should be kept as low as possible. Water quality (if transported in water) and behaviour should be monitored/regulated and noted at regular intervals during and after transport. Water should ideally be aerated with a portable air pump to ensure adequate oxygenation, and if animals are transported in air, they should not be exposed to environmental hypoxia/anoxia.

Land crabs should be transported with some substrate and adequate shelter. Amphibious crabs should not be transported fully immersed in water, but can be transported in a small amount of water, with some substrate (e.g. sediment) to enable air breathing if needed. This can be adjusted depending on the species' level of dependence on water/air breathing and should be detailed in

the application. Decapods are prone to resort to cannibalism when stressed. They could be transported together only if they are transported in large enough containers, with adequate shelter, and over short distances, otherwise they should be separated. The specific transport condition is dependent on the number of animals transported, transport method, transport distance, species and the water quality (especially temperature) of the source/air temperature and the procedure should be well-motivated in each application.

1.4 HANDLING (Code: DHA)

Depending on the species and their size, decapods can be handled using nets and/or hands. Avoid coming into contact with crab pincers, or have your hand wedged between the tail and body of a crayfish/lobster, and wearing gloves may be appropriate. Net mesh sizes should be appropriate. When transferring animals between tanks, water quality parameters (temperature, DO, salinity, pH)/air temperature should be closely matched. An increase in ventilation rates during handling will increase reliance on adequate oxygenation. Animals should be carefully monitored after handling. Handlers who are prone to allergies in general should wear gloves at all times.

1.5 HOLDING (Code: DHO)

Decapods should only be kept at a facility approved by AREC. If decapods are kept at a non-UKZN facility, copies of the facility's relevant permits and/or their Animal Ethics Committee/NSPCA approval must accompany the ethics application.

Facilities should be kept clean and neat. Tanks must be disinfected before and after each experiment and water quality parameters should be kept within the safe limit for the experimental species. Water/room temperature, dissolved oxygen levels (for full aquatic species), salinity and pH must be monitored and recorded daily, while parameters such as ammonia, nitrate, nitrite, dissolved CO_2 and suspended solids should be monitored as needed for the specific experiment/species. Because decapods are ectotherms, the room/water temperature should be regulated. Crabs (and preferably also lobsters/crayfish) should be housed individually to prevent cannibalism. Amphibious crabs should not be immersed in water for the duration of their holding and should be supplied with a perch for air exposure. The same perch (e.g. a dark PVC pipe) can also be used for housing. This is especially important for river and estuarine crabs that may rely extensively on air breathing, but it is important to study the requirements for each species.

Tanks should be cleaned at regular intervals and care should be taken to not unnecessarily disturb animals. Romano and Zeng (2017) highlight some of the factors that increase cannibalism in decapods in aquaculture.

In addition to water/air quality monitoring, animal condition and behaviour should also be monitored daily (See **1.16 & 1.17** for examples of forms). Any

sick animals must be quarantined and treated (see below). Treated animals can only be returned to the holding facility once they are free of pathogens and/or they have regained their health.

1.5.1 Acclimation and quarantine (Code: DAC)

Because newly arrived animals may introduce pathogens into the holding facility, it is essential that new batches of animals are quarantined for an appropriate time before they are integrated in the holding facility. As far as possible, do not mix animals from different sources/collection times. Animals should be carefully monitored during quarantine and all sick animals must be removed and treated in isolation (or euthanised if needed) immediately. Pathogen outbreaks should immediately be reported to AREC as an adverse event.

Animals must be acclimated to the holding facility after transport and quarantine. Experimental procedures should dictate the acclimation time, but acclimation should not be shorter than 24 hours. When bringing new animals to the facility, care should be taken to avoid thermal/osmotic shock. Transport tank conditions should match quarantine tank conditions as closely as possible. If animals are transported in water, transport tank water can be slowly be replaced by receiving tank water in small volumes to allow for thermal and osmotic equilibration. Crabs can be transferred to their holding tanks immediately as long as the air/water temperature matches that of the transport container.

Food should be introduced gradually after transport and animals should be handled as little as possible during acclimation/quarantine.

1.5.2 Record keeping and documentation

The holding facility should have the following documents at hand:

a. Detailed standard operating procedures (SOPs) for collection, transport, quarantine, acclimation, handling and maintenance of equipment;
b. Detailed information of every batch of animals received, such as collection permit; date received, quarantine information, condition, intended use etc.;
c. For each tank: source and date of arrival, estimation of age and weight; the name and contact detail of principal investigators and all other investigators/ students, AREC approval reference number, history of daily water quality measurements and animal condition/ behaviour, morbidity/ mortality/ quarantine detail if appropriate;

Some considerations for health status can be found in Appendix B of the *Canadian Council on Animal Care Guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing*.

Some of the considerations include: feeding activity, physical appearance, clinical signs (if appropriate), provoked behavior and unprovoked behavior (Also see **14.16** for an example).

1.5.3 Food, feeding and nutrition (Code: DFN)

Decapods should ideally be fed commercial feed with the nutritional requirements suitable for the specific species used. The date of manufacture, expiry date and nutritional analysis should be accessible for each batch of food. The food must be stored in airtight containers, in a dark, cool area and kept pest free. Animals must be fed at regular intervals as appropriate for the species and should be fed to satiation. Excess food should be removed from the tanks if appropriate. It may often be necessary to use non-commercial feed. In this case a motivation must be included in the ethics application.

1.6 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. Anaesthetics or sedatives should be used to sedate or immobilise animals in all experiments where decapods are transported, extensively handled or manipulated. Not only does this improve the researcher's ability to handle the animals and perform surgical/invasive procedures, but it also alleviate the stress and pain associated with those procedures.

All anaesthetic procedures should be carried out by properly trained personnel using the appropriate precautions. Animals should be carefully monitored throughout the procedure and during the recovery period in anaesthetic-free water. Animals should then be monitored while recovering overnight.

Decapods can be monitored every five minutes for heart rate (if the equipment is available), righting reflex and taking its defensive posture (in crabs) during anaesthesia and recovery. Because the behaviour of the different species vary so much, it is important to obtain baseline data in healthy individuals before the experiment starts (Minter et al., 2013).

The choice of anaesthetic should take into account the safety of the operators and animals, the level of anaesthesia required, induction and recovery times, the margin between optimal anaesthetic dosage and lethal dosage, as well as the level of anaesthetic-induced stress. Because most of these also depend on water quality, water temperature, decapod species and life-stage used, the anaesthetic of choice should be tested in a small sample of decapod before the onset of experiments.

Some of the chemical anaesthetics suitable for vertebrate anaesthesia are not suitable for decapods, and the choice of anaesthetics (and the concentration) should therefore be carefully considered for each species and motivated.

1.6.1 Chemical anaesthesia

Most chemical anaesthetics block the ventilatory response and it is essential that gills be constantly irrigated. Examples of anaesthesia for decapods are provided below:

1.6.1.1 Immersion anaesthesia (DAIM)

Clove oil:95% ethanol (at 1:2 of clove oil:ethanol) was found to be a suitable immersion anaesthetic for Chinese mitten crabs (*Eriocheir sinensis*) if immersed in water containing 20mL L^{-1} of the clove oil mixture (suitable for a 98 g body size) (Hajek et al., 2009). Recovery in clean water was rapid (~5 minutes). Clove oil can also be used as a prawn anaesthetic, but the concentrations needed to anaesthetise freshwater prawn were 5-10 times higher that that used for fish (Coyle et al., 2004). It is therefore essential to adequately motivate concentrations in the application.

Propiscin and *MS-222* immersion was found not to have the desirable effect on Chinese mitten crabs.

1.6.1.2 Injection anaesthesia (DAIN)

Alfaxalone, a GABA receptor antagonist, was successful for anaesthesia in blue crab (*Calinectes sapidus*), geen crab (*Carcinus maenas*) and brown crab (*Cancer pagurus*). A 15 mg kg^{-1} solution was suitable for a rapid and reliable anaesthetic, although 100 mg kg^{-1} can be used for a long-lasting effect (Minter et al., 2013). The anaesthetic can administer in the pericardial sinus as follows: restrain crabs manually, insert a 25-gauge needle, attached to a 3 mL syringe through the arthrodial membrane between the abdominal and cephalothoracic body segments, advance dorsally until haemolymph is obtained and slowly administer the Alfaxalone (Minter et al., 2013). *Clove oil*, *MS-222*, *procaine, ketamine, xylazine* and *propofol* were found not be suitable. They produced a slow onset of anaesthesia, inconsistent action and distressful responses.

1.6.2 Non-chemical anaesthetics

In some instances, non-chemical anaesthetics might be considered, for example when chemical anaesthetics are known to affect the endpoint measured in the experiment. This is the case with many physiological endpoints, such as plasma hormone levels, lactate and glucose levels. It may also interfere with estimating ectoparasite loads as anaesthetised parasites may detach from the host.

1.6.2.1 Hypothermia (DAHY)

Crabs can be immersed in crashed ice for a short time. For example, it took adult *Portunus sanguinolentus,* (~70g body mass), ~222 sec of immersion to obtain anaesthesia (Premarathna et al., 2016). Hypothermia should not be used for surgery or invasive procedures.

1.7 SAMPLING OF BODY FLUIDS/WASTE PRODUCTS

Appropriate sedation or anaesthesia should be used to restrain crabs for collection of body fluids. It is important to recognise that both restraint and anaesthesia may alter physiological parameters. The potential puncture site should be properly cleaned and prepared to ensure maximum cleanliness.

1.7.1 Bleeding

Blood is an excellent medium for repeated analysis of decapod health and condition. Care should be taken in using an appropriate anti-coagulant, e.g. heparin or EDTA. Blood should be processed in accordance with endpoints measured and stored taking into account the stability of the compounds to be analysed.

1.7.1.1 Bleeding by Cardiac Puncture (Code: DBCP)

Haemolymph can be collected by cardiac puncture from large decapods such as horseshoe crabs by inserting a 14G needle into the hinge between the opistostoma and telson (see Armstrong and Conrad (2008) for a detailed method and visual guide). In lobsters and crabs, where the heart is not accessible by inserting a needle into a hinge between body segments, a small hole can be drilled above the heart and he epidermis carefully removed with small dissection scissors. The small hole in the carapace can be sealed using see-through film, and haemolymph can be collected directly from the heart or the arteries surrounding the heart if accessible. Alternatively, haemolymph can be collected from the drilled hole, without removing a piece of the carapace/epidermis. The hole can be sealed with petroleum jelly to prevent excessive haemolymph loss.

1.7.1.2 Bleeding by accessing a haemolymph cavity (Code: DBHC)

Because decapods have a partially open circulatory system, haemolymph can be collected from any of the cavities filled with haemolymph. In crabs and lobsters haemolymph can be collected by inserting a needle into the arthrodial membrane between the walking legs and the body, or by inserting a needle into the arthordial membrane inbetween the tail segments. The needle can be connected to a syringe, or the haemolymph can be directly collected in a collection tube.

1.7.2 Urine Collection (Code: DUC)

Urine can be collected from two paired nephropores of the antennal gland (at the basis of the eye stalks (Kamio et al., 2014). The nephropores can be lifted with the bent, blunted tip of a needle, and urine can be aspirated with a pipette. Alternatively, the nephropore can be canulated as in Breithaupt et al. (1999).

1.7.3 Faeces Collection (Code: DFC)

Faeces can be collected with a small faecal loop. The loop is inserted in the posterior end of the intestine (Nolan and Smith, 2009). Alternatively, faeces can be syphoned from the holding tank if animals are housed individually.

1.7.4 Carapace and gill booklet scrapings (DGS)

A microscope slice can be scraped over the carapace or gill booklets. A wet mount is then prepared to observe mucus, collected cells and debris for identifying parasites (Nolan and Smith, 2009).

1.8 INJECTIONS

Injections may be required for specific mechanistic studies on signalling, disease development etc. Injections should be carried out with due care and consideration for the welfare of the animal. The needle should be the appropriate size and injection volumes should be as small as possible. Needles should be introduced in spaces between scales where appropriate and injections should be administered under anaesthetic.

Chemicals for injection should ideally be dissolved in sterile physiological saline. Hydrophobic chemicals may be dissolved in very small quantities of ethanol, methanol or DMSO as appropriate as a co-solvent and the pH of chemicals that are not soluble at neutral pH may be slightly adjusted. The osmolarity might have to be adjusted according to the haemolymph osmolarity.

Vehicle/sham injected control animals should form part of the experimental setup.

1.8.1 Injection into the pericardial sinus (Code: DIPS)

Restrain crabs manually, insert a 25-gauge needle, attached to a 3 mL syringe through the arthrodial membrane between the abdominal and cephalothoracic body segments, advance dorsally until haemolymph is obtained and slowly administer the injection (Minter et al., 2013). Alternatively, for continuous injection, a small hole can be drilled at the top of the carapace above the heart and the injection is administered into the pericardial sinus through the whole, which is afterwards sealed with petroleum jelly.

1.9 PHYSIOLOGICAL MEASUREMENTS

This section is by no means exhaustive, since crabs are popular experimental animals. Each physiological measurement should be explained in detail and motivated accordingly in the application. All physiological measurement should minimise stress to the animal and should be conducted by trained individuals.

1.9.1 Respirometry (Code: DPRT)

Measurement of energetics and the response of decapods to experimental conditions is done by quantification of oxygen consumption using a respirometer and gas probe / optode and analyser.

Oxygen consumption rate has become the conventional metabolic measure for decapods because dissolved oxygen can be determined with relative ease and reliability. The iodometric or Winkler method of measuring O_2 concentrations (mg or mL O_2/L water is highly accurate when fresh reagents are available to fix and titrate water samples. Clark-type polarographic electrodes measure O_2 tension or partial pressure (P_{O_2}) and are easier and faster to use than the Winkler method. Newer technologies utilize optodes to assess oxygen content of water. These measurements are carried out in a metabolic chamber, using a either a closed system, a discontinuous open/closed system or a flow-through system using dual probes / optodes to assess P_{O_2} in the incurrent and excurrent water. The P_{O_2} in the chamber should at all times be above the critical P_{O_2} for the species involved.

1.9.2 Measurement of Food Consumption (Code: DPF)

Animals are usually placed in appropriate sized housing and fed for several days before the commencement of the experiment. If the same animals are used in consecutive experiments, they should be given adequate time to recover. Daily weighing of animals, food supplied and eaten will need to be done, and food conversion rates can be calculated.

Invasive body composition measurements of Decapods particularly water and fat content, if required, may only be determined following euthanasia of animals and autopsy / necropsy.

1.9.3 Measurement of Renal Clearance Rate (Code: DPRC)

Animals are weighed and a weight-corrected concentration of a substance (such as inulin) that can ONLY be cleared through the urine is injected into the pericardial sinus. After a set time haemolymph and urine are collected and the volume measured. The inulin concentration of both the haemolymph and urine are measured and the renal clearance rate can be calculated (volume urine produced per minute x inulin conc in urine / inulin conc in haemolymph).

1.9.4 Measurement of Haemolymph volume (Code: DPRC)

Haemolymph volume can be measured by injecting a weight-corrected concentration of inulin into the pericardial sinus (as in 14.9.3). The inulin concentration in a fixed volume of haemolymph is then expressed as a percentage of the body weight, using the equation $v_{heamolymph} = (c_{inulin\ injected} \times v_{inulin\ inulin\ injected} / c_{inulin\ in\ a\ fixed\ volume\ haemolymph}) \times 100$

1.10 SURGICAL PROCEDURES

Surgical procedures in decapods are limited, but each surgical technique should be explained in detail and motivated accordingly in the application. All surgical measurement should minimise stress to the animal and should be conducted under appropriate anaesthesia by trained individuals. SAVC certification is a requirement for conducting surgical procedures on animals, unless the procedures are conducted by a SAVC registered veterinarian.

1.10.1 Laparoscopic Inspection (Code: DSLI)

Access to internal organs can be obtained by removing a small piece of the carapace as described in Nolan and Smith (2009). Laparoscopic inspection under saline can then be performed. Afterwards, the wound must be closed by surgical epoxy or sutures as needed (Nolan and Smith, 2009).

1.10.2 Surgical Biopsy (Code: DSB)

Access to the site for surgical biopsy can be obtained as in 14.10.1., after which biopsies of target tissue can be taken. Typical tissues include gill booklets and carapace. Afterwards, the wound must be closed by surgical epoxy or sutures as needed (Nolan and Smith, 2009).

1.11 EUTHANASIA

When considering euthanasia methods, one must ensure that the animals are euthanised as quickly as possible with minimum fear, pain or suffering, while also considering the health and safety of personnel. A defined endpoint should be established for studies that involve potential pain and/or discomfort. In studies where there is expected morbidity and mortality, the criteria for early euthanasia should be clearly defined in the experimental protocols. Monitoring should be done daily during holding, acclimation and experimentation. Unless morbidity is an approved endpoint of the study, animals should be removed from the experiment before severe morbidity occurs. In experiments where the chances of mortality is high, animals should be monitored more often.

Whilst euthanasia techniques are important to consider, the following considerations are essential: (1) the method of handling animals immediately before and during transfer to the killing facility; (2) handling up to the point of stun; and (3) the immediacy of loss of consciousness. These factors should

be detailed and justified in the research protocol and care should be taken to prevent unnecessary distress to the animals and operator during this process.

The CCAC divides euthanasia techniques into acceptable (immersion and injectable anaesthetic overdose; maceration) and conditionally acceptable (CO_2, concussion, decapitation, cervical dislocation and pithing) euthanasia methods. Their guidelines (*Canadian Council on Animal Care Guidelines on: Euthanasia of animals used in science*.) should be considered in conjunction with the summary below when selecting euthanasia methods appropriate for the study.

1.11.1 Immersion overdose of anaesthetic (Code: DEIO)

Any of the chemicals listed under immersion anaesthesia can be used in overdose. Dosages will be species specific and dependent on the size of the animals.

1.11.2 Injectable anaesthetic (DEIN)

Care should be taken that all injectable anaesthetics are appropriately buffered when used for euthanasia. Any of the injectable anaesthetics can be used for euthanasia if administered as an overdose. Dosages will be species specific and dependent on the size of the animals.

1.11.3 Maceration (Code: DEM)

This form of euthanasia is only suitable for animals smaller than 2cm in length.

1.11.4 Euthanasia in Which Drugs Cannot be Used (Code: PECO)

Any non-chemical means of euthanasia such as pithing is acceptable (Cooper, 2011). The need for alternative methods must be justified in the research protocol. CO_2 by itself is no longer recognised as an acceptable method of euthanasia, and should CO_2 be used for euthanasia, it must be combined with other acceptable gasses such as halothane or Isofor (Isoflurane).

1.12 NECROPSY (DN)

Necropsy and tissue/organ harvesting must be conducted using the appropriate sterile techniques and protection. Specimens must be stored appropriately and all biological waste should be discarded appropriately according to biological waste protocols, and incinerated. Basic preparation for histopathology can be found in Nolan and Smith (2009)

1.13 RELEASE OF DECAPODS INTO THE WILD (Code: DR)

In general, research animals that have been kept in captive environments must not be released into the wild. Release into the wild is only permissible under appropriate licence from the relevant authority/ies.

1.14 STUDIES ON KNOWN PATHOGENS OF DECAPODS

Animals may only be inoculated with known pathogens under a special permit issued by the National Department of Agriculture, Forestry and Fisheries: Animal Health Directorate when planning such studies. In addition, these studies may only be conducted in facilities that are able to act as appropriate containment facilities.

1.15 DISPOSAL OF DEAD ANIMALS

Carcasses must be disposed of according to acceptable national and municipal regulations for the disposal of biological materials, or in accordance with permit conditions in the case of aquaculture facilities.

1.16 EXAMPLES OF HEALTH EVALUATION SHEETS

1.16.1 Monitoring of groups of decapods (per tank) or individuals during holding/acclimation/experiments

SPECIES				**ANIMAL NO:**			
HOLDING/EXPERIMENT							
AREC NO.							
NOTE NORMAL AGRESSION/RIGHTING BEHAVIOUR:							
DATE							
TIME							
UNDISTURBED OBSERVATION	Scoring 4 (Excellent) to 1 (Poor) **						
Normal defensive behaviour (If applicable)							
Feeding behaviour							
Adequate food intake							
Body mass stable/increases							
No evidence of parasites/infection							
No evidence of lesions on body							
No evidence of growths on body							
Apendages intact							
Regular movement							
No signs of lethargy							
Responsive to external stimuli (eye stalk test)							
No. of mortalities							
No. of animals moved to quarantine/back into holding							
No. of animals euthanised							
OTHER							
SIGNATURE							

Special Requirements:
Animals can be monitored once a day during holding/acclimation, but more often during experiments/quarantine.
* Each application must detail considerations for setting humane endpoints. Sick animals must be moved into quarantine and observed frequently. If condition does not improve, euthanasia should be considered, based on the endpoints set. Animals showing obvious signs of distress during experiments should be removed into control conditions. If the adverse effects do not improve, euthanasia should be considered, based on the endpoints set.

1.16.2 Monitoring individual animals during experiments/quarantine

SPECIES				ANIMAL NO:			
HOLDING/EXPERIMENT							
AREC NO.							
NOTE NORMAL AGRESSION/RIGHTING BEHAVIOUR:							
DATE							
TIME							
UNDISTURBED OBSERVATION	Scoring 4 (Excellent) to 1 (Poor) **						
Normal defensive behaviour (if applicable)							
Normal righting behaviour (if applicable)							
Feeding behaviour							
Adequate food intake							
Body mass stable/increases							
No evidence of parasites/infection							
No evidence of lesions on body							
No evidence of growths on body							
Apendages intact							
Regular movement							
No signs of lethargy							
Responsive to external stimuli (eye stalk test)							
No. of mortalities							
No. of animals moved to quarantine/back into holding							
No. of animals euthanised							
OTHER							
SIGNATURE							

Special Requirements:
Animals can be monitored once a day during holding/acclimation, but more often during experiments/quarantine.
* Each application must detail considerations for setting humane endpoints. Sick animals must be moved into quarantine and observed frequently. If condition does not improve, euthanasia should be considered, based on the endpoints set. Animals showing obvious signs of distress during experiments should be removed into control conditions. If the adverse effects do not improve, euthanasia should be considered, based on the endpoints set.

1.17 EXAMPLE OF WATER QUALITY SHEET (if needed)

SPECIES				**TANK NO**			
HOLDING/EXPERIMENT							
AREC NO.							
DATE							
TIME							
MEASUREMENTS	**REFERENCE RANGE****						
Temperature							
Water replacement rate							
pH							
Ammonia*							
Salinity							
Dissolved oxygen level							
NOTES							
AREAS OF CONCERN							
ACTION TAKEN							
SIGNATURE							

* NOT NEEDED DAILY – TWICE A WEEK IS SUFFICIENT
** REFERENCE RANGES ARE SPECIES DEPENDANT

1.18 REFERENCES

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2 STANDARD PROTOCOLS FOR CEPHALOPODS

2.1 OVERVIEW

Cephalopoda is a class in the phylum Mollusca. Cephalopods are therefore not vertebrates, but they employ discrimination learning to avoid pain (Mather, 2001; Takuwa-Kuroda et al., 2003) and they are considered to be the have the most advanced brain of all invertebrates (Takuwa-Kuroda et al., 2003). Ethical approval is therefore needed to conduct research on cephalopods at UKZN. The class Cephalopoda include nautiloids, sepiolids (cuttlefish), squids and octopuses. No South African cephalopods are harmful to humans.

2.1.1 Useful guideline documents

This guideline is not exhaustive and all methods/procedures should be suitable for the specific species/experimental conditions/endpoints measured and must be supported by evidence from literature. Some detailed methods/procedures can be found in Fiorito et al. (2015).

All research involving collecting cephalopods are subject to international, national and provincial legislation and policies covering collection and transport of animals (please see section 10 in the *AREC Guide to the Care and Use of Animals in Research and Teaching*). The necessary permits and permission need to be obtain prior to the start of the project as per AREC guidelines, including State Veterinary certificates for movement of animals where needed. The local fisheries/conservation officers must at all times be notified prior to collection of wild decapods, and care should be taken not to collect protected species, unless a permit has been obtained.

2.1.2 Selection of Experimental Procedures/Endpoints

When using live animals in research one must aim to select endpoints appropriate for the outcomes of the experiment, whilst minimising pain and distress to the animal. In addition, endpoints indicating discomfort, pain and morbidity should also be defined and humane endpoints for euthanasia should be pre-determined. The *Canadian Council on Animal Care Guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing* provides detailed guidelines.

Some specific points:

- Animals should be carefully monitored during acclimation and experimentation and a table similar to that in **2.16 & 2.17** can be used to record animal health parameters. All observations should be conducted by trained personnel.

- A small pilot study might be required to test whether the endpoints, number of animals (via power analysis) and observation frequency selected are suitable for the experiment. This also allows for training of personnel.

- While the Organisation for Economic Cooperation and Development (OECD) discourages acute toxicology studies with death as an endpoint (i.e. standard lethal toxicity testing), well-motivated studies will be considered, if the OECD protocols (www.oecd.org) are closely followed to minimise animal numbers and all possible care is taken to relieve animal discomfort, pain and distress.

- Studies involving the exposure of cephalopods to environmental extremes should select the earliest endpoint possible.

The least stressful procedures should at all times be selected and all procedures must aim to inflict minimum harm, morbidity and mortality. All procedures must only be carried out by competent individuals. All para-veterinarian procedures must be carried out under supervision/in consultation of the University veterinarian. Veterinary procedures may only be carried out by a registered veterinarian. South African Veterinary Council (SAVC) certification/authorisation may be a requirement for certain procedures and it is best to consult with Animal Care technicians in in the Biomedical Resources Unit/the resident veterinarian during the planning phase of the project.

2.2 CAPTURE (Code: OC)

There are multiple methods for collection of cephalopods. It is recommended that cephalopods be collected using basket traps for cuttlefish, jigs for squids and pots for octopuses. Alternatively, cephalopods can be hand collected, with care, from pools, under rocks or underwater by SCUBA diving at certain locations. The exact methods should be adequately outlined and supported by literature. It is important to note possible effects of the collection method on the animal's health, or that of non-target taxa. Since all cephalopods have soft delicate skin, they are easily damaged by mechanical abrasion. Therefore, the capture of juvenile and adult cephalopods from the wild results in high mortality, and considerable care must be taken if viable organisms are to be returned to a laboratory.

2.3 TRANSPORT (Code: OT)

Cephalopods should be transported only once necessary permits/veterinary certificates are in place. Transport conditions should inflict as little stress as possible. The container should be of the appropriate size, well insulated to prevent changes in temperature and light intensity, and should not leak. The container should be disinfected before use, and water quality must match the appropriate water quality standards. Care should also be taken to match water quality between the water body where animals are collected from, the

transport container, and the tank in which animals will be kept in the laboratory to avoid osmotic/thermal shock. Because cephalopods are ectotherms, it might be appropriate to use slightly cooler water during transport to lower metabolism. If possible, decapods should be fasted for 12-48 hours prior to transport and this time will depend on species, age and water quality. This minimizes the contribution of faeces to ammonia loading of the water. Ammonia-nitrogen levels should be kept as low as possible. Water quality (if transported in water) and behaviour should be monitored/regulated and noted at regular intervals during and after transport. Water should ideally be aerated with a portable air pump to ensure adequate oxygenation, and because animals have delicate skin, they should remain moist at all times.

The specific transport condition is dependent on the number of animals transported, transport method, transport distance, species and the water quality (especially temperature) of the source/air temperature and the procedure should be well-motivated in each application.

Transport of animals should always be in seawater. The levels of available oxygen and accumulation of metabolites in a limited volume are important considerations for transport of living cephalopods. Wherever possible and applicable to research, the transport of eggs is the simplest and preferable approach. Small cephalopods can be placed in buckets part-filled with seawater, which must be changed with regularity to avoid substantially altering temperature, pH and oxygen content. Cephalopods that are captured at sea should be kept in deck tanks filled with seawater continuously pumped from the ocean. Transport of cephalopods should be minimised, and where possible the researcher should travel to study the cephalopods not vice versa. For some species, like octopuses, stronger transport containers should be used as they are occasionally reported to cut and bite through thin plastic bags. When cephalopods are being transported, the potential impact upon their health and welfare will need to be assessed and careful consideration given to the time required for acclimation before experimentation.

2.4 HANDLING (Code: OHA)

As previously mentioned cephalopods are very delicate, and extreme effort must be made to avoid skin damage and bruising. Since the skin is easily damaged on dry surfaces, cephalopods of all types must be kept continuously moist and exposed to air for minimal periods. They should therefore be handled only when necessary. The number of handling episodes should also be limited and handling procedures should only be carried out by competent individuals using techniques that minimize the potential for injury. Restraint

and handling of cephalopods should be carried out in a manner to minimize visual stimulation. Where feasible, cephalopods should be protected from direct sunlight and rapid changes in lighting while being restrained.

When transferring animals between tanks, water quality parameters (temperature, DO, salinity, pH)/air temperature should be closely matched. An increase in ventilation rates during handling will increase reliance on adequate oxygenation. Animals should be carefully monitored after handling. Handlers who are prone to allergies in general should wear gloves at all times.

2.5 HOLDING (Code: OHO)

Cephalopods should only be kept at a facility approved by AREC. If they are kept at a non-UKZN facility, copies of the facility's relevant permits and/or their Animal Ethics Committee/NSPCA approval must accompany the ethics application. It is important to understand that cephalopods require large volumes of water and the facilities should be capable of maintaining aeration and filtration if the normal system fails.

Facilities should be kept clean and neat. Tanks must be disinfected before and after each experiment and water quality parameters should be kept within the safe limit for the experimental species. Water temperature, dissolved oxygen levels, salinity and pH must be regulated within the physiological range reported for each species. It should be monitored, using calibrated equipment, and recorded daily. Parameters such as ammonia, nitrate, nitrite, dissolved CO_2 and suspended solids should be monitored as needed for the specific experiment/species. The accumulation of potentially toxic nitrogenous compounds can cause problems, particularly in closed systems, and must therefore be monitored. A build-up of nitrogenous compounds can lead to behavioural changes and/or in skin colouration in cephalopods. It is necessary to monitor and avoid accumulation of ink (for example: when keeping cuttlefish in large volumes). The tank flow rates should also be adjusted when there is increased inking.

Cephalopod housing systems are predominantly based on open systems where a continuous supply of fresh seawater from a nearby location is available. Artificial seawater preparations are also considered adequate and contain all the necessary substances and trace elements to keep cephalopods in good health. This includes any mixture designed for marine invertebrates and corals but not fish. Trace elements, in particular strontium and calcium, should be monitored and added, if necessary. Tanks should be

cleaned at regular intervals and care should be taken to not unnecessarily disturb animals.

In addition to water quality monitoring, animal condition and behaviour should also be monitored daily (See **2.16 & 2.17** for examples of forms). Any sick animals must be quarantined and treated (see below). Treated animals can only be returned to the holding facility once they are free of pathogens and/or they have regained their health.

Photoperiod and light intensity should be maintained according to the natural living habits and possibly the geographical origin of the cephalopod species. The use of a weak ambient light (e.g. moonlight lamp) or a specific red light illumination reduces the risk of disturbance when observation of the animal is required at night.

Noise, vibration and other sources of disturbance should be avoided. Octopuses should be given plenty of shelter to use as 'homes'. Gravel, pebbles and stones are recommended in order to facilitate self-construction of a shelter for cephalopods. Smooth, curved walls are recommended for cuttlefish and squid. Environmental enrichment can be achieved in the tank environment using varying factors including the shape of the tank, flow of water, and a variety of prey items, providing opportunities for the cephalopods to engage in specific activities.

2.5.1 Acclimation and quarantine (Code: OAC)

Because newly arrived animals may introduce pathogens into the holding facility, it is essential that new batches of animals be quarantined for an appropriate time before they are integrated in the holding facility. As far as possible, do not mix animals from different sources/collection times. Animals should be carefully monitored during quarantine and all sick animals must be removed and treated in isolation (or euthanised if needed) immediately. Pathogen outbreaks should immediately be reported to AREC as an adverse event.

Irrespective of their origin, all animals must be acclimated to the holding facility after transport and quarantine. Experimental procedures should dictate the acclimation time, but acclimation should not be shorter than 24 hours. Cephalopods are stenotherm and stenohaline, and it is important to avoid thermal/osmotic shock when brining new animals to the facility. Transport tank conditions should match quarantine tank conditions as closely as possible. If animals are transported in water, transport tank water can be slowly be replaced by receiving tank water in small volumes to allow for thermal and

osmotic equilibration.

Food should be introduced gradually after transport and animals should be handled as little as possible during acclimation/quarantine. Evidence suggests that for cuttlefish and octopus, an adequate predatory performance is considered a good sign of acclimatisation to a tank.

2.5.2 Record keeping and documentation

The holding facility should have the following documents at hand:

d. Detailed standard operating procedures (SOPs) for collection, transport, quarantine, acclimation, handling and maintenance of equipment;
e. Detailed information of every batch of animals received, such as collection permit; date received, quarantine information, condition, intended use etc.;
f. For each tank: source and date of arrival, estimation of age and weight; the name and contact detail of principal investigators and all other investigators/ students, AREC approval reference number, history of daily water quality measurements and animal condition/ behaviour, morbidity/ mortality/ quarantine detail if appropriate;

Cephalopods must be inspected at least once a day by a competent person. Some considerations for health status can be found in Fiorito et al. (2015)(Also see **15.18** for an example).Signs of health and illness in cephalopods vary with species. Signs based on appearance, behaviour and physiology include:

a. Abnormal body colouration/body patterning;
b. skin texture including; lesions, swelling, bruising, erosions and ulceration;
c. abnormal morphology or damage to cuttlebone or shell;
d. abnormal body posture or position in the tank;
e. reduced food intake (and subsequent weight loss);
f. reduced social behaviour (i.e.: lack of response, refusal to leave shelter or a sluggish response);
g. reduced grooming;
h. abnormal motor or loco motor coordination;
i. excessive inking; and
j. abnormal or changes in ventilation.

2.5.3 Food, feeding and nutrition (Code: OFN)

Most cephalopods are carnivores and actively hunt, while nautiloids are scavengers. Some octopuses will eat dead food. Live feed is therefore the best feed for some species, but synthetic/artificial feed is suitable for some.

The feeding requirements of cephalopods are complex, and differ between species. Fiorito et al. (2015) provides a useful summary of different aspects to take into account.

If artificial/synthetic food is used, the date of manufacture, expiry date and nutritional analysis should be accessible for each batch of food. The food must be stored in airtight containers, in a dark, cool area and kept pest free. Animals must be fed at regular intervals as appropriate for the species and should be fed to satiation. Excess food should be removed from the tanks if appropriate. If live feed is used, a motivation must be included in the ethics application.

2.6 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. Anaesthetics or sedatives should be used to sedate or immobilise animals in all experiments where animals are transported, extensively handled or manipulated. Not only does this improve the researcher's ability to handle the animals and perform surgical/invasive procedures, but it also alleviates the stress and pain associated with those procedures.

Properly trained personnel, using the appropriate precautions, should carry out all anaesthetic procedures, and special attention should be paid to preserving cardio-respiratory function. Animals should be carefully monitored throughout the procedure and during the recovery period in anaesthetic-free water. Animals should then be monitored while recovering overnight.

The choice of anaesthetic should take into account the safety of the operators and animals, the level of anaesthesia required, induction and recovery times, the margin between optimal anaesthetic dosage and lethal dosage, as well as the level of anaesthetic-induced stress.

Some of the chemical anaesthetics suitable for vertebrate anaesthesia might not be suitable for cephalopods, and the choice of anaesthetics (and the concentration) should therefore be carefully considered for each species and motivated. Because most of these also depend on water quality, water temperature, experimental species and life-stage used, the anaesthetic of choice should be tested in a small sample of animals before the onset of experiments. Cardio-respiratory function should be maintained at all times, while protocols for resuscitating cephalopods (e.g. mantle massage) should be available. As far as possible, blood pressure, heart rate, respiration rates should be monitored (ideally non-invasively) during anaesthesia.

The commonly used anaesthetics allow maintenance of anaesthesia for up to 30 minutes in cephalopods. The animal should be rendered unconscious and insensitive to painful stimuli. Guidelines for the assessment of anaesthesia include:

a. Depression of ventilation, sometimes accompanied by reduced cardiac activity;
b. Decrease in chromatophore tone (indicates a reduced drive to or from the sub-oesophageal chromatophore lobes);
c. Reduced arm activity, tone and sucker adhesion;
d. Loss of normal posture and righting reflex;
e. Reduced or absent response to a noxious stimulus (Andrews et al., 2013).

Most anaesthetics block the ventilatory response and it is essential that gills be constantly irrigated. Examples of anaesthesia for cephalopods are provided below:

2.6.1 Immersion anaesthesia (OAIM)

Most techniques use *magnesium chloride*, *ethanol* or *benzocaine* (Andrews et al., 2013; Scimeca, 2011), but *clove oil* has also been considered (Andrews et al., 2013).

2.6.2 Injection anaesthesia (OAIN)

All injection substances should be dissolved in the appropriate medium (physiological saline for hydrophilic substances and ethanol or DMSO for hydrophobic substances). The concentration of the anaethetic should be high enough to inject he smallest possible volume and should be administered by trained personnel. It is worth noting that DMSO in itself can be toxic, so the lowest possible DMSO concentration should be used to dissolve the anaesthetic substance. All experiments should be accompanied by a vehicle/sham control.

Various routes of administration exist in Cephalopods (see detail in Fiorito et al. (2015), Table 9). Possible injection sites are:

a. Cardiac injection into the branchial hearts;
b. Dorsal aorta or the afferent branchial vessel through a canula implanted under anaesthesia;
c. Intramuscular at the base of the arm;
d. In the arm in the region of the branchial nerve to provide nerve block;
e. In the cephalic vein;

f. Into the vertical brain lobe;
g. Into the crop/stomach.

Anaesthetics include: *magnesium chloride with an ethanol push* injected into the branchial hearts or ventral brain lobe; or *ethanol* injected into the branchial vessel, web tissue at the base of the arms, cephalic vein or fin nerve branch.

Magnesium chloride with an ethanol push can be used to obtain surgical plain anaesthesia (Scimeca, 2011).

2.6.3 Non-chemical anaesthetics

In some instances, non-chemical anaesthetics might be considered, for example when chemical anaesthetics are known to affect the endpoint measured in the experiment. This is the case with many physiological endpoints, such as plasma hormone levels, lactate and glucose levels. It may also interfere with estimating ectoparasite loads as anaesthetised parasites may detach from the host.

2.7 SAMPLING OF BODY FLUIDS/WASTE PRODUCTS

Appropriate sedation or anaesthesia should be used to restrain cephalopods for collection of body fluids. It is important to recognise that both restraint and anaesthesia may alter physiological parameters. The potential puncture site should be properly cleaned and prepared to ensure maximum cleanliness.

2.7.1 Bleeding (OB)

Haemolymph (blood) is an excellent medium for repeated analysis of animal health and condition. Care should be taken in using an appropriate anti-coagulant, e.g. heparin or EDTA. Blood should be processed in accordance with endpoints measured and stored taking into account the stability of the compounds to be analysed.

In cephalopods haemolymph can be collected from branchial hearts (Code: OBBH), branchial vessels (Code: OBBV), cephalic vessels between the eyes (Code OBCV) or via a catheter implanted under anaesthetic into the anterior vena (Code: OBAV) (For detail on the species and needle size, see Fiorito et al. (2015), Table 9).

2.7.2 Urine Collection (Code: OUC)

Urine can be collected with a syringe from the renal sac post mortem by cutting the medial septum and inverting the mantle (Sakamoto et al., 2015).

2.7.3 Ink collection (OIC)

Ink can be collected post mortem using one of two methods: (1) collect ink from the duct end of the ink sac with a syringe, with as little manipulation of the ink sac as possible, or (2) dissect out the ink sac and milk its contents out into a tube by running forceps along its length. This method can cause the sample to be contaminated by damaged tissue (Derby, 2014).

2.8 INJECTIONS

Injections may be required for specific mechanistic studies on signalling, disease development etc. For example, alcian blue can be injected into cephalopods for marking, or antibiotics can be administered by injection. Injections should be carried out with due care and consideration for the welfare of the animal. The needle should be the appropriate size and injection volumes should be as small as possible. Needles should be introduced in spaces between scales where appropriate and injections should be administered under anaesthetic.

Chemicals for injection should ideally be dissolved in sterile physiological saline. Hydrophobic chemicals may be dissolved in very small quantities of ethanol, methanol or DMSO as appropriate as a co-solvent and the pH of chemicals that are not soluble at neutral pH may be slightly adjusted. The osmolarity might have to be adjusted according to the haemolymph osmolarity.

Vehicle/sham injected control animals should form part of the experimental setup.

Detail on injection in cephalopods can be found in Fiorito et al. (2015), Table 9.

Substances can be injected into the web tissue at the arm base (Code: OIAB), into the side of the neck at a depth of 10 mm (Code: OISN), into the cephalic vein (Code: OICV), intramuscular at the base of the arm, in the head or into the mantle (Code: OIIM), into the fin nerve branch (Code: OIFN), Into the brain vertical lobe (Code: OIBV), into the crop/stomach (OICS), into the brachial hear (Code: OIBH), into the dorsal aorta via a cannula implanted under anaesthesia (Code: OIDA).

2.9 PHYSIOLOGICAL MEASUREMENTS

This section is by no means exhaustive.

2.9.1 Respirometry (Code: DPRT)

Measurement of energetics and the response of animals to experimental conditions is done by quantification of oxygen consumption using a respirometer and gas probe / optode and analyser.

Oxygen consumption rate has become the conventional metabolic measure for aquatic animals because dissolved oxygen can be determined with relative ease and reliability. The iodometric or Winkler method of measuring O_2 concentrations (mg or mL O_2/L water is highly accurate when fresh reagents are available to fix and titrate water samples. Clark-type polarographic electrodes measure O_2 tension or partial pressure (P_{O_2}) and are easier and faster to use than the Winkler method. Newer technologies utilize optodes to assess oxygen content of water. These measurements are carried out in a metabolic chamber, using a either a closed system, a discontinuous open/closed system or a flow-through system using dual probes / optodes to assess P_{O_2} in the incurrent and excurrent water. The P_{O_2} in the chamber should at all times be above the critical P_{O_2} for the species involved.

Measures of growth rates (Code: OPG), feeding rates (Code: OPFR) and feed conversion rates (Code: OPFC) are discussed in (Wells and Clarke, 1996).

2.10 SURGICAL PROCEDURES

Surgical procedures in cephalopods are discouraged, since they are generally not well-described in literature, and are not as advanced as in vertebrates in terms of monitoring physiological parameters, control of haemorrhage (cephalopod haemolymph do not have clotting factors and because haemolymph is clear/blue, it is difficult to identify a haemorrhage), controlled anaesthesia, wound closure and post-operative infection control. If surgery is essential to the project, the following consideration must be motivated as part of the ethics application:

a. Ethical justification relating to harm vs. benefits;
b. Identification of potential adverse events and steps taken to refine procedures to limit these;
c. How post-operative suffering will be minimized;
d. Clearly define humane endpoints (Fiorito et al., 2015).

All procedures need to be detailed in the application. SAVC certification is a requirement for conducting surgical procedures on animals, unless the procedures are conducted by a SAVC registered veterinarian.

2.11 EUTHANASIA

When considering euthanasia methods, one must ensure that the animals are euthanised as quickly as possible with minimum fear, pain or suffering, while also considering the health and safety of personnel. A defined endpoint should be established for studies that involve potential pain and/or discomfort. In studies where there is expected morbidity and mortality, the criteria for early euthanasia should be clearly defined in the experimental protocols.

Monitoring should be done daily during holding, acclimation and experimentation. Unless morbidity is an approved endpoint of the study, animals should be removed from the experiment before severe morbidity occurs. Various physical, behavioural and clinical signs are used to evaluate the health of cephalopods and should be used to pre-et endpoints that would warrant removal of the animal from the experiment or that would warrant euthanasia (See section 15.24). The researcher should take responsibility for cephalopod welfare during the senescence process, ensuring that animals are humanely killed unless there is scientific or animal welfare justification for their remaining alive. In experiments where the chances of mortality are high, animals should be monitored more often.

Whilst euthanasia techniques are important to consider, the following considerations are essential: (1) the method of handling animals immediately before and during transfer to the killing facility; (2) handling up to the point of stun; and (3) the immediacy of loss of consciousness. These factors should be detailed and justified in the research protocol and care should be taken to prevent unnecessary distress to the animals and operator during this process.

The CCAC divides euthanasia techniques into acceptable (immersion and injectable anaesthetic overdose; maceration) and conditionally acceptable (CO_2, concussion, decapitation, cervical dislocation and pithing) euthanasia methods. Their guidelines (*Canadian Council on Animal Care Guidelines on: Euthanasia of animals used in science.*) should be considered in conjunction with the summary below when selecting euthanasia methods appropriate for the study.

2.11.1 Immersion overdose of anaesthetic (Code: OEIO)

Anaesthetic overdose is considered the most humane method for euthanising cephalopods (Andrews et al., 2013). Any of the chemicals listed under immersion anaesthesia (magnesium chloride/ethanol) can be used in immediate overdose, or by gradually increasing the concentration. Dosages will be species specific and dependent on the size of the animals, and Andrews et al. (2013) provides some examples of concentrations and time-to-death. It is suggested that immersion overdose is followed by a physical method such as rapid decapitation (see below). Chloroform is not recommended, since it produces an initial violent reaction. It is not acceptable to kill hatchlings or larvae by immersing them directly into tissue fixative – they should rather be euthanised with immersion anaesthetic first.

2.11.2 Euthanasia in Which Drugs Cannot be Used (Code: PECO)

2.11.2.1 Cooling

Cold seawater containing 2% ethanol has been used before, but should be followed by a physical method.

2.11.2.2 Other

Euthanasia with maceration is not advised, since it may cause undue stress and avoidable suffering. CO_2 by itself is no longer recognised as an acceptable method of euthanasia, and should CO_2 be used for euthanasia, it must be combined with other acceptable gasses such as halothane or Isofor (Isoflurane).

The need for all alternative methods should to be justified in the research protocol. All the methods above must be followed by a procedure to confirm death. This includes destruction of the brain, exsanguination or maceration in small animals (detail in Andrews et al. (2013))

2.12 NECROPSY (ON)

Necropsy and tissue/organ harvesting must be conducted using the appropriate sterile techniques and protection. Specimens must be stored appropriately and all biological waste should be discarded appropriately according to biological waste protocols, and incinerated.

2.13 RELEASE OF CEPHALOPODS INTO THE WILD (Code: OR)

In general, research animals that have been kept in captive environments must not be released into the wild. Release into the wild is only permissible under appropriate licence from National Department of Agriculture, Forestry and Fisheries (DAFF).

2.14 STUDIES ON KNOWN PATHOGENS OF DECAPODS

Animals may only be inoculated with known pathogens under a special permit issued by DAFF: Animal Health Directorate when planning such studies. In addition, these studies may only be conducted in facilities that are able to act as appropriate containment facilities.

2.15 DISPOSAL OF DEAD ANIMALS

Carcasses must be disposed of according to acceptable national and municipal regulations for the disposal of biological materials, or in accordance with permit conditions in the case of aquaculture facilities.

2.16 EXAMPLES OF HEALTH EVALUATION SHEETS

2.16.1 Monitoring of cephalopods during holding/acclimation/experiments

SPECIES				**ANIMAL NO:**			
HOLDING/EXPERIMENT							
AREC NO.							
NOTE NORMAL PHYSICAL/BEHAVIOUR/CLINICAL ENDPOINTS: Detail can be found in Andrews et al. (2013) and Fiorito et al. (2015), but specific baseline parameters must be defined for each species in advance.							
DATE							
TIME							
UNDISTURBED OBSERVATION	Scoring 4 (Excellent) to 1 (Poor) **						
Normal skin colour (no abnormal patterning)							
Normal skin texture (no unexplained swellings/growths)							
Normal skin integrity (no tears/ulcers/cysts)							
Normal body posture							
No evidence of parasites/infection							
Normal feeding/hunting behaviour							
No uncontrolled defaecation/inking/vomiting							
Normal food intake							
No abnormal motor/locomotor coordination							
No signs of lethargy							
Normal social behaviour for social species							
No. of mortalities							
No. of animals moved to quarantine/back into holding							
No. of animals euthanised							
OTHER							
SIGNATURE							

Special Requirements:
Animals can be monitored once a day during holding/acclimation, but more often during experiments/quarantine.
* Each application must detail considerations for setting humane endpoints. Sick animals must be moved into quarantine and observed frequently. If condition does not improve, euthanasia should be considered, based on the endpoints set. Animals showing obvious signs of distress during experiments should be removed into control conditions. If the adverse effects do not improve, euthanasia should be considered, based on the endpoints set.

2.16.2 Monitoring individual animals during experiments/quarantine

SPECIES				**ANIMAL NO:**			
HOLDING/EXPERIMENT							
AREC NO.							
NOTE NORMAL PHYSICAL/BEHAVIOUR/CLINICAL ENDPOINTS: Detail can be found in Andrews et al. (2013) and Fiorito et al. (2015), but specific baseline parameters must be defined for each species in advance.							
DATE							
TIME							
UNDISTURBED OBSERVATION	Scoring 4 (Excellent) to 1 (Poor) **						
Normal skin colour (no abnormal patterning)							
Normal skin texture (no unexplained swellings/growths)							
Normal skin integrity (no tears/ulcers/cysts)							
Normal body posture							
No evidence of parasites/infection							
Normal feeding/hunting behaviour							
No uncontrolled defaecation/inking/vomiting							
Normal food intake							
No abnormal motor/locomotor coordination							
No signs of lethargy							
Normal social behaviour for social species							
No. of mortalities							
No. of animals moved to quarantine/back into holding							
No. of animals euthanised							
OTHER							
SIGNATURE							

Special Requirements:
Animals can be monitored once a day during holding/acclimation, but more often during experiments/quarantine.
* Each application must detail considerations for setting humane endpoints. Sick animals must be moved into quarantine and observed frequently. If condition does not improve, euthanasia should be considered, based on the endpoints set. Animals showing obvious signs of distress during experiments should be removed into control conditions. If the adverse effects do not improve, euthanasia should be considered, based on the endpoints set.

2.17 EXAMPLE OF WATER QUALITY SHEET (if needed)

SPECIES				**TANK NO**			
HOLDING/EXPERIMENT							
AREC NO.							
DATE							
TIME							
MEASUREMENTS	**REFERENCE RANGE****						
Temperature							
Water replacement rate							
pH							
Ammonia*							
Salinity							
Dissolved oxygen level							
NOTES							
AREAS OF CONCERN							
ACTION TAKEN							
SIGNATURE							

* NOT NEEDED DAILY – TWICE A WEEK IS SUFFICIENT
** REFERENCE RANGES ARE SPECIES DEPENDANT

2.18 REFERENCES

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3 STANDARD PROTOCOLS FOR FISH

3.1 OVERVIEW

As vertebrates, fish must be treated with the same respect afforded to other vertebrates and as outlined in the *AREC Guide to the Care and Use of Animals in Research and Teaching*. For the purposes of this guideline, fishes include all agnathous fishes (hagfish and lampreys), cartilaginous fishes and bony fishes. As specified in the *AREC Guide to the Care and Use of Animals in Research and Teaching* fish eggs, embryos or larvae that have not developed beyond exclusive reliance on their yolk nutrients are not covered in this guideline.

3.1.1 Useful guideline documents

This guideline is not exhaustive and all methods/procedures should be suitable for the specific species/experimental conditions/endpoints measured and must be supported by evidence from literature. This document should also be read together with the SANS 10386:2008 document, and specifically Appendix G, which deals with the care and management of fish. Some detailed methods/procedures can be found in:

Canadian Council on Animal Care Guidelines on: the care and use of fish in research, teaching and testing.
http://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf

Canadian Council on Animal Care Guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing.
http://www.ccac.ca/Documents/Standards/Guidelines/Appropriate_endpoint.pdf

Canadian Council on Animal Care Guidelines on: Euthanasia of animals used in science.
http://www.ccac.ca/Documents/Standards/Guidelines/Euthanasia.pdf

Canadian Council on Animal Care Guidelines on: Fish anaesthetics.
http://www.ccac.ca/Documents/Standards/Guidelines/Add_PDFs/Fish_Anesthetics.pdf

All research involving collecting fish are subject to international, national and provincial legislation and policies covering collection and transport of fish (please see section 10 in the *AREC Guide to the Care and Use of Animals in Research and Teaching*). The necessary permits and permission need to be obtain prior to the start of the project as per AREC guidelines, including State Veterinary certificates for movement of animals where needed. The local fisheries/conservation officers must at all times be notified prior to collection of wild fish.

3.1.2 Selection of Experimental Procedures/Endpoints

When using live animals in research one must aim to select endpoints appropriate for the outcomes of the experiment, whilst minimising pain and distress to the animal. In addition, endpoints indicating discomfort, pain and morbidity should also be defined and humane endpoints for euthanasia should be pre-determined. The *Canadian Council on Animal Care Guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing* provides detailed guidelines.

Some specific points:

- Animals should be carefully monitored during acclimation and experimentation and a table similar to that in **3.16 & 3.17** can be used to record animal health parameters. All observations should be conducted by trained personnel.

- A small pilot study might be required to test whether the endpoints, number of animals (via power analysis) and observation frequency selected are suitable for the experiment. This also allows for training of personnel.

- While the Organisation for Economic Cooperation and Development (OECD) discourages acute toxicology studies with death as an endpoint (i.e. standard lethal toxicity testing), well-motivated studies will be considered, if the OECD protocols (www.oecd.org) are closely followed to minimise animal numbers and all possible care is taken to relieve animal discomfort, pain and distress.

- Studies involving the exposure of fish to environmental extremes should select the earliest endpoint possible.

The least stressful procedures should at all times be selected and all procedures must aim to inflict minimum harm, morbidity and mortality. All procedures must only be carried out by competent individuals. All para-verterinarian procedures must be carried out under supervision/in consultation of the University veterinarian. Veterinary procedures may only be carried out by a registered veterinarian. South African Veterinary Council (SAVC) certification may be a requirement for certain procedures and it is best to consult with Animal Care technicians in in the Biomedical Resources Unit/the resident veterinarian during the planning phase of the project.

3.2 CAPTURE (Code: PC)

There are multiple methods for collection of fish, including electrofishing, netting (cast nets, gill nets, hand nets) as well as traditional fishing techniques. The collection method should match the species collected and the environment from which animals are collected (e.g. ocean/estuary/river/dam/hatchery). These methods should be adequately outlined and supported by literature. It is important to note that possible effects of the collection method on the animal's health, or non-target taxa. For example, electrofishing are stressful and also affect non-target species. Gill

netting for example can damage gills and cause hypoxia/infection. Nets with an appropriate mesh size should be used at all times, and excessive weight loading at the bottom of nets should be avoided. Where possible barbless hooks should be used when the hook and line method is employed.

3.3 TRANSPORT (Code: PT)

Fish should be transported only once necessary permits/veterinary certificates are in place. Transport conditions should inflict as little stress as possible. The container should be of the appropriate size, well insulated to prevent changes in temperature and light intensity, and should not leak. The container should be disinfected before use, and water quality must match the appropriate water quality standards. Care should also be taken to match water quality between the water body where animals are collected from, the transport container, and the tank in which animals will be kept in the laboratory to avoid osmotic/thermal shock. Because fish are poikilotherms, it might be appropriate to use slightly cooler water during transport to lower metabolism. If possible, fish should be fasted for 12-48 hours prior to transport and this time will depend on species, age and water quality. This minimizes the contribution of faeces to ammonia loading of the water. Ammonia-nitrogen levels should be kept as low as possible. Water quality and behaviour should be monitored and noted at regular intervals during and after transport. Water should ideally be aerated with a portable air pump to ensure adequate oxygenation.

The transport condition is dependent on the number of fish transported, transport method, transport distance, species and the water quality (especially temperature) of the source and the procedure should be well-motivated in each application.

3.4 HANDLING (Code: PHA)

Fish can be handled using nets and/or hands. Aquatic animals are slippery and if hands are used it is best to use both hands and care should be taken that fish do not slip from the hands with sudden movement. The handler should at all times avoid removing the protective mucous coat or scales, and should prevent abrasions, as it serves as a natural barrier to infection. Air exposure and changes in ambient temperature should be avoided or kept to a minimum. If possible, fish should be caught in hand nets individually to avoid excessive weight loading and injury. Net mesh sizes should be appropriate. When transferring fish between tanks, water quality parameters (temperature, DO, salinity, pH) should be closely matched. An increase in ventilation rates during handling will increase reliance on adequate water oxygenation. Fish should be carefully monitored after handling.

It is worth mentioning that some fish have sharp/spiny dorsal fins and the use of gloves may be necessary. Avoid the use of latex/nitrile gloves, since they may become slippery when wet, which reduces the grip on the animal.

Handlers with allergies to fish or are prone to allergies in general should wear gloves at all times.

3.5 HOLDING (Code: PHO)

Fish should only be kept at a facility approved by AREC. If fish are kept at a non-UKZN facility, the ethics application must be accompanied a copies of the facility's relevant permits and/or their Animal Ethics Committee/NSPCA approval.

Facilities should be kept clean and neat. Tanks must be disinfected before and after each experiment and water quality parameters should be kept within the safe limit for the experimental species. Water temperature, dissolved oxygen levels, salinity and pH must be monitored and recorded daily, while parameters such as ammonia, nitrate, nitrite, dissolved CO_2 and suspended solids should be monitored as needed for the specific experiment/species. Fish should ideally not be exposed to a temperature change of more than 2°C every 12 hours, and dissolved oxygen levels should be kept above 90% saturation for most species. Water pH must be kept at optimal levels for the species, and if pH decreases, water ammonia levels should be measured to ensure that ammonia levels remain below the toxic level. Tanks should be cleaned at regular intervals and care should be taken to not unnecessarily disturb fish. Fish do get accustomed to human interaction during routine operations, but some species are naturally more skittish, and this should be taken into consideration when routine operations are performed.

Fish should be kept at an acceptable stocking density for the species, and tank conditions must be adjusted as appropriate for each species. In addition to water quality monitoring, fish condition and behaviour should also be monitored daily (See **13.17** & **13.18** for examples of forms). Any sick fish must be quarantined and treated (see below). Treated fish can only be returned to the holding facility once they are free of pathogens and/or they have regained their health.

3.5.1 Acclimation and quarantine (Code: PAC)

Because newly arrived fish may introduce pathogens into the holding facility, it is essential that new batches of fish are quarantined for an appropriate time before they are integrated in the holding facility. As far as possible, do not mix fish from different sources/collection times. Fish should be carefully monitored during quarantine and all sick fish must be removed and treated in isolation (or euthanised if needed) immediately. Pathogen outbreaks should immediately be reported to AREC as an adverse event.

Fish must be acclimated to the holding facility after transport and quarantine. Experimental procedures should dictate the acclimation time, but acclimation

should not be shorter than 24 hours. When bringing new fish to the facility, care should be taken to avoid thermal/osmotic shock. Transport tank conditions should match quarantine tank conditions as closely as possible. If fish were transported in plastic bags, the bags can be floated in the receiving tank to equilibrate temperature, and receiving tank water can be added to the plastic bag in small volumes to allow for osmotic equilibration. Similarly, transport tank water can be slowly be replaced by receiving tank water in small volumes to allow for thermal and osmotic equilibration. If however the fish arrive in poor quality water, they must be transferred to the receiving tank immediately and the monitoring period increased.

Food should be introduced gradually after transport and fish should be handled as little as possible during acclimation/quarantine.

3.5.2 Record keeping and documentation (Code: PRKD)

The holding facility should have the following documents at hand:

g. Detailed standard operating procedures (SOPs) for collection, transport, quarantine, acclimation, handling and maintenance of equipment;
h. Detailed information of every batch of fish received, such as collection permit; date received, quarantine information, condition, intended use etc.;
i. For each tank: source and date of arrival, estimation of age and weight; the name and contact detail of principal investigators and all other investigators/ students, AREC approval reference number, history of daily water quality measurements and fish condition/ behaviour, morbidity/ mortality/ quarantine detail if appropriate;

Some considerations for health status can be found in Appendix B of the *Canadian Council on Animal Care Guidelines on: Choosing an appropriate endpoint in experiments using animals for research, teaching and testing.* Some of the considerations include: feeding activity, physical appearance, clinical signs (if appropriate), provoked behavior and unprovoked behavior (Also see **13.18** for an example).

3.5.3 Food, feeding and nutrition (Code: PFN)

Fish should ideally be fed commercial feed with the nutritional requirements suitable for the specific species used. The date of manufacture, expiry date and nutritional analysis should be accessible for each batch of food. The food must be stored in airtight containers, in a dark, cool area and kept pest free. Fish must be fed at regular intervals as appropriate for the species and should be fed to satiation. Excess food should be removed from the tanks if appropriate. It may often be necessary to use non-commercial feed. In this case a motivation must be included in the ethics application.

3.6 ANAESTHESIA

Anaesthesia, including tranquillisation and post-operative analgesia, needs to be appropriate for each individual procedure. Anaesthetics or sedatives should be used to sedate or immobilise fish in all experiments where fish are transported, extensively handled or manipulated. Not only does this improve the researcher's ability to handle the fish and perform surgical/invasive procedures, but it also alleviate the stress and pain associated with those procedures.

All anaesthetic procedures should be carried out by properly trained personnel using the appropriate precautions. Fish should be carefully monitored throughout the procedure and during the recovery period in anaesthetic-free water. Personnel should be cognisant of the three main stages of anaesthesia and recovery and fully understand potential reactions to the anaesthetic (*Canadian Council on Animal Care Guidelines on: Fish anaesthetics*):

STAGES OF ANAESTHESIA	**DESCRIPTION**
I	Loss of equilibrium
II	Body immobilised with continued opercular movement
III	Body immobilised and opercular movement stopped
STAGES OF RECOVERY	**DESCRIPTION**
I	Opercular movements started, but body still immobilised
II	Regular opercular movements and gross body movements started
III	Pre-anaesthetic appearance and equilibrium regained

The choice of anaesthetic should take into account the safety of the operators and animals, the level of anaesthesia required, induction and recovery times, the margin between optimal anaesthetic dosage and lethal dosage, as well as the level of anaesthetic-induced stress. Because most of these also depend on water quality, water temperature, fish species and life-stage used, the anaesthetic of choice should be tested in a small sample of fish before the onset of experiments.

The *Canadian Council on Animal Care Guidelines on: Fish anaesthetics* provides an extensive review of chemical and non-chemical anaesthetics suitable for use on a for a variety of test fish species, the dosages used, induction times and recovery times as summarised below (please see the document for full detail). In summary:

3.6.1 Chemical anaesthesia

Most chemical anaesthetics block the ventilatory response and it is essential that gills are constantly irrigated.

3.6.1.1 Immersion anaesthesia (PAIM)

Tricaine methyl sulphonate (TMS/MS-222/Metacaine/Tricaine/Finquel) is the most widely used anaesthetic used for fish, safe to use, readily soluble in water and are allowed for use in fish destined for consumption once it has been fully cleared (5-7 days). Because it allows for a deep anaesthesia and blunts the involuntary muscle reflex it is regarded as the most suitable anaesthetic for surgical and invasive procedures (Also Collymore, et al. 2014). It has short induction times and a short recovery period. It may however affect hematocrit, cortisol and lactate levels and may therefore not be suitable for all physiological studies. Adequate buffering prevents many of the undesired effects due to changes in medium pH.

Benzocaine is mostly harmless to humans, has a short induction time and short recovery time. It is not suitable in procedures involving surgery as fish may maintain locomotory movement during all stages of anaesthesia.

Lindocaine is generally safe to use, but adequate buffering with sodium bicarbonate is needed to ensure small variation in effective dosage. It has a very short induction time and a reasonable recovery time.

Clove oil has a short induction time and short recovery time (but the recovery time is longer than for the same concentration of TMS), Although it is safe to use it does not block the involuntary muscle reflex, which makes it unsuitable for surgery and invasive procedures.

2-Phenoxyethanol is a mild toxicant and may cause skin irritation as well as liver and kidney damage in humans and should therefore be handled with care. There is also a small margin between the effective dose and lethal dose in fish, and it could cause an increase in plasma cortisol, glucose and lactate levels within the first 24 hours post exposure. It is also unsuitable for surgery since it does not block the nerve reflex. This all makes it a less than ideal anaesthetic for fish.

Other anaesthetics that may be considered include *Metomidate, Propoxate, Quinaldine sulphate* and *Propanidid.* The following anaesthetics are unsuitable for use in fish: *Chlorobutanol, Diethyl ether* and *Chloral hydrate.*

3.6.1.2 Injection anaesthesia (PAIN)

Ketamine hydrochloride is safe to handle and is widely use in human and

veterinary medicine. It has a short induction time, but long recovery time. Because it does not block ventilatory rhythm, it is suitable for long-term anaesthesia where one cannot maintain constant irrigation of gills.

3.6.2 Non-chemical anaesthetics

In some instances, non-chemical anaesthetics might be considered, for example when chemical anaesthetics are known to affect the endpoint measured in the experiment. This is the case with many physiological endpoints, such as plasma hormone levels, lactate and glucose levels. It may also interfere with estimating ectoparasite loads as anaesthetised parasites may detach from the host.

3.6.2.1 Hypothermia (PAHY)

Hypothermia is achieved by lowering the water temperature to between 10 and 25°C, or to 0°C for fish acclimated to water above 10°C. This can be accomplished by immersing fish into an ice slurry, but the hypothermia method is not appropriate for temperate fish. The use of dry ice as coolant should be avoided as the high levels of CO_2 can result in hypercapnia and acidic conditions in the water and are harmful if inhaled by the handler in poorly ventilated environments.

3.6.2.2 Electroanaethesia (PAEA)

Electro anaesthesia is suitable to immobilising fish during collection and tagging. It has few long-term deleterious effects, but can cause increases in plasma glucose and corticosteroids. It also affects non-target taxa, such as invertebrates when used in field collection.

3.7 SAMPLING OF BODY FLUIDS/WASTE PRODUCTS

Appropriate sedation or anaesthesia should be used to restrain fish for collection of body fluids. It is important to recognise that both restraint and anaesthesia may alter physiological parameters. The skin and other sites should be properly cleaned and prepared to ensure maximum cleanliness.

3.7.1 Bleeding

Blood is an excellent medium for repeated analysis of fish health and condition. Care should be taken in using an appropriate anti-coagulant, e.g. heparin or EDTA. Blood should be processed in accordance with endpoints measured, e.g. for haematocrit, collecting plasma, serum, whole blood or dry blood spots, and stored taking into account the stability of the compounds to be analysed.

3.7.1.1 Bleeding by Cardiac Puncture (Code: PBCP)

In instances where euthanasia and destructive tissue sampling is indicated by the protocol, blood collection by cardiac puncture may be considered, either by

accessing the heart directly after thoracic opening, or directly through the skin using appropriately gauge / length needles. Cardiac puncture for routine sampling should be discouraged.

3.7.1.2 Bleeding by Caudal Venepuncture (Code: PBCV)

Blood can be collected by caudal venepuncture from anaesthetised or freshly euthanised fish. Blood is collected by inserting a needle connected to a syringe/vacutainer under the skin at the ventral midline of the caudal peduncle.

3.7.1.3 Bleeding by Dorsal Aortic Puncture (Code: PBDAP)

Blood can be collected by dorsal aortic puncture from anaesthetised or freshly euthanised fish. Blood is collected by inserting a needle connected to a syringe/vacutainer, bevel point up, along the dorsal midline of the mouth, just past the juncture of the second gill arch.

3.7.1.4 Bleeding by Dorsal Aortic Cannulation (Code: PBDAC)

Cannulation provides opportunity for obtaining repeated blood samples from the same fish without handling. Repeated blood sampling via cannula should be restricted to fish greater than 150 g. Cannulae should be inserted under surgical anaesthesia by trained personnel.

Preferred locations for cannulation are the dorsal and ventral aortae, due to ease of access via the opercular cavity, and extension of cannulae externally via the opercular cavity or nostril (See Tashjian and Hung, (2005) for detail).

3.7.1.5 Bleeding by Terminal Procedures (Code: PBTB)

Collecting blood from small fish is problematic, and tail ablation preceded by euthanasia is recommended. The caudal peduncle is severed with a scalpel or sharp knife, and blood is collected in a haematocrit tube, or by pipette.
Ablation allows blood collection directly from the dorsal aorta of small fish.

3.7.2 Urine Collection (Code: PUC)

Urine can be collected from the urinary bladder by internal or external catheters (Tashjian and Hung, 2005; Curtis and Wood, 1991).

3.7.3 Faeces Collection (Code: PFC)

The fish should be removed from the anaesthetic solution, blotted dry on the underside and stripped of faeces by applying gentle pressure to the abdomen between the anal fin and the anus. As soon as the faeces have been expelled fish should be returned to clean water and observed for normal behaviour.

3.8 INJECTIONS

Injections may be required for specific mechanistic studies on signalling,

disease development etc. Injections should be carried out with due care and consideration for the welfare of the fish. The needle should be the appropriate size and injection volumes should be as small as possible. Needles should be introduced in spaces between scales where appropriate and injections should be administered under anaesthetic.

Chemicals for injection should ideally be dissolved in sterile physiological saline. Hydrophobic chemicals may be dissolved in very small quantities of ethanol, methanol or DMSO as appropriate as a co-solvent and the pH of chemicals that are not soluble at neutral pH may be slightly adjusted.

Vehicle/sham injected control fish should form part of the experimental setup.

3.8.1 Subcutaneous injection (SQ) (Code: PISQ)

The needle should be introduced in spaces between scales. This form of injection may however lead to the formation of sterile abscesses.

3.8.2 Intramuscular injection (IM) (Code: PIIM)

Intramuscular injections may be made into the large dorsal epaxial and abdominal muscles, taking care to avoid the lateral line and ventral blood vessels.

3.8.3 Intraperitoneal (IP)(Code: PIIP)

Intraperitoneal (IP) injections should avoid penetrating abdominal viscera as substances that cause inflammation may lead to adhesion formation.

3.8.4 Intra-cardiac (IC) (Code: PIIC)

This procedure is not advised in fish because of the high mortality rate due to massive blood loss, infection and fatal organ failure when the heart is punctured. If the need for intra-cardiac injection is well motivated, qualified individuals should carry this out.

3.8.5 Retro-orbital (RO) (Code: PIRO)

This procedure provides a suitable alternative to intravenous and intra-cardiac injection. Substances are introduced into the retro-orbital venous sinus just behind the eye. This method is suitable for drug delivery and hemapoietic stem cell transplantation (Pugach et al., 2009).

3.9 PHYSIOLOGICAL MEASUREMENTS

This section is by no means exhaustive, since fish are popular experimental animals. Each physiological measurement should be explained in detail and motivated accordingly in the application. All physiological measurement

should minimise stress to the animal and should be conducted by trained individuals.

3.9.1 Respirometry (Code: PPRT)

Measurement of energetics and the response of fish to experimental conditions is done by quantification of oxygen consumption using a respirometer and gas probe / optode and analyser.

Oxygen consumption rate has become the conventional metabolic measure for fish because dissolved oxygen can be determined with relative ease and reliability. The iodometric or Winkler method of measuring O_2 concentrations (mg or mL O_2/L water is highly accurate when fresh reagents are available to fix and titrate water samples. Clark-type polarographic electrodes measure O_2 tension or partial pressure (P_{O_2}) and are easier and faster to use than the Winkler method. Newer technologies utilize optodes to assess oxygen content of water. These measurements are carried out in a metabolic chamber, using a either a closed system, a discontinuous open/closed system or a flow-through system using dual probes / optodes to assess P_{O_2} in the incurrent and excurrent water. The P_{O_2} in the chamber should at all times be above the critical P_{O_2} for the fish species involved.

The end-point with greatest functional significance to fish health, condition and performance is the critical swimming speed, U_{crit}. This is derived in a swim-tunnel or analogous setup where the fish is challenged with a step-wise increase in water flow rate, until loss of position is observed. Subsequently, fish are allowed to recover under observation (see 13.1.2 and 13.17).

The functional equivalent of U_{crit} is the aerobic scope. For the assessment of aerobic scope, a fish is encouraged, by touch with a gloved hand, to swim until exhaustion (evidenced by lack of movement when touched in tail region). The fish is immediately transferred to a respirometer and the maximal post-exercise oxygen consumption rate measured (see above). Aerobic scope is derived as the difference between maximal and resting oxygen consumption rates, and predicts the ability of the fish to compensate for environmental of biological change. Subsequently, fish are allowed to recover under observation (see 13.1.2 and 13.17).

3.9.2 Measurement of Food Consumption (Code: PPF)

Animals are usually placed in appropriate sized housing and fed for several days before the commencement of the experiment. If the same animals are used in consecutive experiments, fish should be given adequate time to recover. Daily weighing of animals, food supplied and eaten will need to be done.

Invasive body composition measurements of fish particularly water and fat content, if required, may only be determined following euthanasia of animals and autopsy / necropsy.

Non-invasive assessment of body condition can be derived by pressure differential, where fish are housed in an appropriately sized pressure chamber. Water pressure increase (corresponding to a 3 m depth change) is induced with a syringe, and the fish compensates by swim bladder adjustment. The degree of swim bladder adjustment is offset by body fat content, and forms the basis for the assessment. Measurements are completed in ~5 min, and can be carried out repeatedly on the same fish. Subsequently, fish are allowed to recover under observation (see 13.1.2 and 13.17).

3.9.3 Activity measurements (Code: PPA)

Behavioural assessment provides useful information on environmental selection by fish. Selection criteria, e.g. thermal or salinity preference, should be within the range normally experienced by the species under investigation. Observations should be recorded (visually, manually or through videography) without altering the behaviour of the fish.

3.10 SURGICAL PROCEDURES

This section is by no means exhaustive, since fish are popular experimental animals and a variety of techniques are used. Each surgical technique should be explained in detail and motivated accordingly in the application. All surgical measurement should minimise stress to the animal and should be conducted under appropriate anaethesia by trained individuals. SAVC certification is a requirement for conducting surgical procedures on animals, unless the procedures are conducted by a SAVC registered vetrinarian.

3.10.1 Laparoscopic Inspection (Code: PSLI)

This method provides a fast, minimally invasive method for determining brood stock sex/organ health and should be used in large fish only. A detailed method can be found in Falahatkar et al. (2011).

3.10.2 Surgical Biopsy or Organectomy (Code: PSB)

Surgical biopsies may be needed if the equipment for laparoscopic inspection is not available. Surgical gonadectomy can be used to prevent sexual maturation, or in hormone-treatment studies. An example of a method can be found in Brown et al. (1982). The precise method should be detailed in the application.

3.11 EUTHANASIA

When considering euthanasia methods for fish, one must ensure that the fish

is euthanised as quickly as possible with minimum fear, pain or suffering, while also considering the health and safety of personnel. A defined endpoint should be established for studies that involve potential pain and/or discomfort. In studies where there is expected morbidity and mortality, the criteria for early euthanasia should be clearly defined in the experimental protocols (see CCAC guidelines). Monitoring should be done daily during holding, acclimation and experimentation. Unless morbidity is an endpoint of the study, fish should be removed from the experiment before severe morbidity occurs. In experiments where the chances of mortality is high, fish should be monitored more often.

Whilst euthanasia techniques are important to consider, the following considerations are essential: (1) the method of handling fish immediately before and during transfer to the killing facility; (2) handling up to the point of stun; and (3) the immediacy of loss of consciousness. These factors should be detailed and justified in the research protocol and care should be taken to prevent unnecessary distress to the fish and operator during this process.

The CCAC divides euthanasia techniques into acceptable (immersion and injectable anaesthetic overdose; maceration) and conditionally acceptable (Concussion, decapitation, cervical dislocation and pithing) euthanasia methods. Their guidelines (*Canadian Council on Animal Care Guidelines on: Euthanasia of animals used in science.*) should be considered in conjunction with the summary below when selecting euthanasia methods appropriate for the study.

3.11.1 Immersion overdose of anaesthetic (Code: PEIO)

The CCAC recommends TMS for immersion euthanasia by overdose, but it should be buffered adequately. Benzocaine, ethomidate, methomidate and clove oil may also be deemed suitable. Immersion euthanasia should always be followed by a chemical or physical method to induce brain death. This method of euthanasia may be ineffective in fish that breath-hold or that breathes air.

3.11.2 Injectable anaesthetic (PEIN)

Care should be taken that all injectable anaesthetics (especially barbiturates and TMS) are appropriately buffered when used for euthanasia. Full details on dosages can be obtained in the CCAC guidelines and the use of drug/dosage must be fully described and motivated in the application.

3.11.3 Maceration (Code: PEM)

This form of euthanasia is only suitable for fish smaller than 2cm in length.

3.11.4 Euthanasia in Which Drugs Cannot be Used (Code: PECO)

Other non-chemical means of euthanasia such as concussion, decapitation, cervical dislocation and pithing are deemed acceptable in the 2013 Report of the AVMA Panel on Euthanasia. The need for alternative methods must be justified in the research protocol and concussion should always be followed by a secondary technique such as cervical dislocation or pithing to ensure that the animal is brain dead. CO_2 by itself is no longer recognised as an acceptable method of euthanasia, and should CO_2 be used for euthanasia, it must be combined with other acceptable gasses such as halothane or Isofor (Isoflurane).

3.12 NECROPSY/TISSUE HARVESTING (PN)

Necropsy and tissue/organ harvesting must be conducted using the appropriate sterile techniques and protection. Specimens must be stored appropriately and all biological waste should be discarded appropriately according to suitable biological waste protocols, and incinerated.

3.13 RELEASE OF FISH INTO THE WILD (Code: PR)

In general, research fish that have been kept in captive environments must not be released into the wild. Release into the wild is only permissible under appropriate licence from the relevant authority.

3.14 TAGGING OF FISH IN THE WILD (Code: PT)

The CCAC guidelines on animal care describe in detail the use of tagging fish in the wild. Animal health during handling and tagging should be considered. The size shape and colour of the tags should be taken into consideration and should not hamper the animal's normal behaviour and physiology. If needed, a small pilot study should be run in the lab to optimise tag properties for specific species. The size, shape and weight of implanted telemetry devices such as PIT tags should also be considered and not interfere with normal physiology and behaviour, and infection of the implant site should be avoided.

3.15 STUDIES ON GENETICALLY MODIFIED FISH

Genetically modified fish is defined as: fish where the genetic code has been altered through genetic engineering, but not through artificial selection of natural traits (Phillips, 2008). When designing experiments on genetically modified (transgenic) fish one should consider the legislation and the local Conservation agency's policies on keeping genetically modified fish, and obtain the necessary permits. One should also outline the considerations for keeping an animal where the physiology, behaviour and immunology can differ significantly from that of the wild-type. Containment is a very important point to consider, especially when working with aquatic organisms. Applicants should also be aware that ethical approval might be subject to approval by the UKZN Institutional Biosafety Committee (IBC).

3.16 STUDIES ON KNOWN FISH PATHOGENS

Fish may only be inoculated with known fish pathogens under a special permit issued by the National Department of Agriculture, Forestry and Fisheries: Animal Health Directorate when planning such studies. In addition, these studies may only be conducted in facilities that are able to act as appropriate containment facilities.

3.17 DISPOSAL OF DEAD FISH

Fish must be disposed of according to acceptable national and municipal regulations for the disposal of biological materials, or in accordance with permit conditions in the case of aquaculture facilities.

3.18 EXAMPLES OF HEALTH EVALUATION SHEETS

3.18.1 Monitoring of groups of fish (per tank) during holding/acclimation/experiments

SPECIES				**TANK NO:**			
HOLDING/EXPERIMENT							
AREC NO.							
NOTE NORMAL SWIMMING BEHAVIOUR/POSITION IN WATER COLUMN/ACTIVITY LEVELS/SCHOOLING BEHAVIOUR FOR THIS SPECIES:							
DATE							
TIME							
UNDISTURBED OBSERVATION	Scoring 4 (Excellent) to 1 (Poor)						
Normal swimming behaviour							
Normal posture in water column							
Feeding behaviour							
Adequate food intake							
No evidence of white spots on body/external parasites							
No evidence of lesions on body							
No evidence of growths on body							
Fins intact							
Regular opercular movement							
No signs of gasping for air at the surface or obvious escape behaviour							
No. of mortalities							
No. of fish moved to quarantine*							
No. of fish euthanised							
Reason for quarantine							
Additional notes							
SIGNATURE							

Special Requirements:
Animals can be monitored once a day during holding/acclimation, but more often during experiments/quarantine.
* Each application must detail considerations for setting humane endpoints. Sick fish must be moved into quarantine and observed frequently. If condition does not improve, euthanasia should be considered, based on the endpoints set. Animals showing obvious signs of distress during experiments should be removed into control conditions. If the adverse effects do not improve, euthanasia should be considered, based on the endpoints set.

3.18.2 Monitoring individual fish during experiments/quarantine

SPECIES				ANIMAL NO:			
HOLDING/EXPERIMENT							
AREC NO.							
NOTE NORMAL SWIMMING BEHAVIOUR/POSITION IN WATER COLUMN FOR THIS SPECIES:							
DATE							
TIME							
UNDISTURBED OBSERVATION	Scoring 4 (Excellent) to 1 (Poor) **						
Normal swimming behaviour							
Normal position in water column							
Feeding behaviour							
Adequate food intake							
Body mass stable/increases							
No evidence of white spots on body/external parasites							
No evidence of lesions on body							
No evidence of growths on body							
Fins intact							
Regular opercular movement							
No signs of gasping for air at the surface or obvious escape behaviour							
Responsive to external stimuli							
Clear cornea							
No signs of ataxia/convulsions							
No. of mortalities							
No. of fish moved to quarantine/back into holding							
No. of fish euthanised							
OTHER							
SIGNATURE							

Special Requirements:
Animals can be monitored once a day during holding/acclimation, but more often during experiments/quarantine.
* Each application must detail considerations for setting humane endpoints. Sick fish must be moved into quarantine and observed frequently. If condition does not improve, euthanasia should be considered, based on the endpoints set. Animals showing obvious signs of distress during experiments should be removed into control conditions. If the adverse effects do not improve, euthanasia should be considered, based on the endpoints set.

3.19 EXAMPLE OF WATER QUALITY SHEET

SPECIES				**TANK NO**			
HOLDING/EXPERIMENT							
AREC NO.							
DATE							
TIME							
MEASUREMENTS	**REFERENCE RANGE****						
Temperature							
Water replacement rate							
pH							
Ammonia*							
Salinity							
Dissolved oxygen level							
NOTES							
AREAS OF CONCERN							
ACTION TAKEN							
SIGNATURE							

* NOT NEEDED DAILY – TWICE A WEEK IS SUFFICIENT
** REFERENCE RANGES ARE SPECIES DEPENDANT

3.20 REFERENCES

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