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TITOLO TESI

"Effect of different formulations of magnesium chloride, used as anaesthetic agents, on the performance of the isolated heart of *Octopus vulgaris"*

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Sommario

Il cloruro di magnesio ($MgCl_2$) rappresenta l'anestetico più comunemente utilizzato per i cefalopodi; tuttavia, i suoi effetti fisiologici compresi quelli a livello cardiaco non sono ben caratterizzati. Nel presente studio, utilizzando come modello sperimentale il polpo comune *Octopus vulgaris,* è stato valutato mediante la tecnica del cuore isolato e perfuso: a) se l'esposizione dell'animale *in vivo* (immersione) a diverse formulazioni di $MgCl_2$ induce effetti sulla funzione cardiaca *in vitro* che potrebbero danneggiare il recupero dall'anestesia; b) l'effetto diretto della perfusione con diverse formulazioni di $MgCl_2$ sulla funzione cardiaca.

Per gli esperimenti *in vivo,* sono state utilizzate le seguenti formulazioni con relativi tempi di esposizione: *i)* $MgCl_2$ 3,5% in acqua di mare ($MgCl_2$ sw, 20min); *ii)* $MgCl_2$ 3,5% (1:1, acqua di mare:acqua distillata, 20min); miscela di $MgCl_2$ (1,12%) + etanolo (1%) (Mix, 20min e 45min); ipotermia (4°C, 5-10min).

Alle condizioni basali, i cuori prelevati dopo esposizione ai vari trattamenti anestetici, inclusa l'ipotermia, sono stati in grado di lavorare con carichi emodinamici simili a quelli *in vivo* e di produrre valori fisiologici di gittata sistolica.

In condizioni di carico emodinamico (incrementi di precarico), i cuori rimossi da animali esposti *in vivo* ad $MgCl_2$ sw (20min) o al Mix (20min) hanno mostrato una performance cardiaca (in termini di risposta di Frank-Starling) comparabile a quella di animali anestetizzati mediante ipotermia. Al contrario, sia l'esposizione a $MgCl_2$ (1:1, 20min), che al Mix (45min) ha mostrato un significativo deterioramento della risposta di Frank-Starling in termini di ridotta capacità di rispondere agli incrementi di precarico.

Dopo esposizione ad ipotermia, la perfusione dei cuori isolati con concentrazioni crescenti delle diverse formulazioni di $MgCl_2$, ha prodotto un effetto bradicardico dose-dipendente (e in alcuni casi arresto cardiaco) ed una riduzione della gittata cardiaca e del volume sistolico, indicativi di un effetto diretto dei trattamenti anestetici sul cuore. Al contrario, la perfusione con dosi crescenti di solo etanolo non ha causato alcuna variazione della frequenza cardiaca, indicando che l'effetto bradicardico evidenziato con la soluzione Mix è attribuibile al cloruro di magnesio.

L'espressione di HSP70 e caspasi-3, e la fosforilazione di JNK e p38MAPK sono stati determinati in omogenati cardiaci, ottenuti da esemplari di *O. vulgaris* esposti alle diverse formulazioni di cloruro di magnesio o ad ipotermia (4°C). I risultati hanno evidenziato un

incremento dell'espressione di HSP70 (ma non della caspasi-3); in particolare, l'espressione di HSP70 è risultata essere inferiore nei cuori prelevati da animali esposti ad ipotermia, rispetto a quelli esposti alle diverse formulazioni di $MgCl_2$. Tra questi ultimi, nessuna differenza quantitativa è stata evidenziata tra le diverse formulazioni, ad eccezione del Mix 45min, effetto dovuto probabilmente al maggior tempo di esposizione.

L'aumento della fosforilazione di JNK e p38MAPK, parallelo all'aumentata espressione di HSP70, lascia supporre il coinvolgimento di queste MAPKs nell'espressione di HSP70.

Nell'insieme i dati dimostrano che, a condizione che l'esposizione *in vivo* al 3,5% di $MgCl_2$ in acqua di mare o ad una miscela di $MgCl_2$+etanolo sia limitato a ~ 20min, gli effetti residui sulla funzione cardiaca non influenzano il recupero post-anestesia.

Inoltre, l'espressione di HSP70 che può giocare un ruolo citoprotettivo nella risposta allo stress durante l'anestesia, parallela alla mancata espressione della caspasi-3 in tutti i tessuti analizzati, permette di escludere eventi pro-apoptotici da parte di tutti i trattamenti anestetici.

Nell'insieme questi risultati possono contribuire alla discussione circa le pratiche anestesiologiche da utilizzare anche in relazione all'applicazione della nuova Direttiva 2010/63 / UE per i cefalopodi.

Summary

Magnesium chloride ($MgCl_2$) is a commonly used for inducing anaesthesia in cephalopods, but its physiological effects including those at cardiac level are not well characterised. We used an *in vitro* isolated perfused systemic heart preparation from the common octopus, *Octopus vulgaris*, to investigate: a) if *in vivo* exposure to $MgCl_2$ formulations had an effect on cardiac function *in vitro* and, if so, could this impact recovery from anaesthesia; b) direct effects of $MgCl_2$ formulations on cardiac function.

For *in vivo* experiments, the following formulations (and relative exposure times) have been used: i) $MgCl_2$ 3.5% in sea water ($MgCl_2$ sw, 20min); ii) $MgCl_2$ 3.5% (1: 1, sea water: distilled water, 20min); mixture of $MgCl_2$ (1.12%) and ethanol (1%) (Mix, 20min and 45min); Hypothermia (4 ° C, 5-10 min).

At baseline conditions the hearts removed after exposure to various anesthetic treatments, including hypothermia, were able to work with hemodynamic loads similar to those *in vivo* and to produce physiological values of stroke volume.

In vitro hearts removed from animals exposed *in vivo* to 3.5% $MgCl_2$ in sea water (20min) or to a mixture of $MgCl_2$ +ethanol (1.12/1%; 20min) showed cardiac function (heart rate, stroke volume, cardiac output) comparable to hearts removed from animals killed under hypothermia. However, 3.5% $MgCl_2$ (1:1, sea water: distilled water, 20min) and 45min of exposure to the $MgCl_2$ + ethanol mixture produced a significant impairment of the Frank-Starling response.

After exposure to hypothermia, perfusion of the isolated heart with $MgCl_2$ ±ethanol formulations produced a concentration-related bradycardia (and arrest), a decreased stroke volume and cardiac output indicating a direct effect on the heart. On the contrary, the heart perfusion with increasing doses of ethanol alone caused no change in heart rate, indicating that the bradycardic effect highlighted with the Mix solution is accounted to a direct effect of magnesium chloride on heart.

The expression of HSP70 and caspase-3, and the phosphorylation of JNK and p38MAPK were determined on cardiac homogenates obtained from specimens of *O. vulgaris* exposed to different formulations of magnesium chloride or hypothermia (4 ° C). The results showed an increased expression of HSP70 (but not of caspase-3); in particular, the

expression of HSP70 was found to be lower in the hearts isoleted from animals exposed to hypothermia, compared to those exposed to the various formulations of $MgCl_2$. Among magnesium chloride formulations, no quantitative difference was found, with the exception of Mix 45min, most probably due to the effect of exposure time.

The increased phosphorylation of JNK and p38MAPK, parallel to the increased expression of HSP70 may suggest the involvement of these MAPKs in the expression of HSP70.

Overall, provided that the *in vivo* exposure to 3.5% $MgCl_2$ in sea water or to a mixture of $MgCl_2$ +ethanol is limited to ~20min, residual effects on cardiac function are unlikely to impact post-anaesthetic recovery. Furthermore, the expression of HSP70, which can play a cytoprotective role in the stress response during anaesthesia, parallel to the lack of expression of caspase-3 in all the analyzed tissues, it allows to exclude events pro-apoptotic by all anesthetics treatments .

Taken together, these results can contribute to the discussion about the anesthetic practices to be used also in relation to the application of the new Directive 2010/63 / EU for cephalopods.

1. Introduction

1.1 General anaesthesia

The word "anaesthesia" has a Greek derivation, meaning loss of sensation or insensibility. Sedation is a preliminary state of anaesthesia (**Fig. 1**) caracterized by a drowsiness state with dulled sensory perception and with some analgesia (insensitivity to pain), but in which there is no loss of sensory perception or of equilibrium (Ross and Ross, 2008).

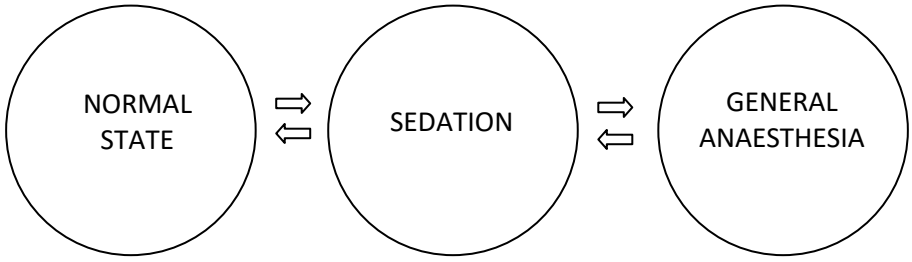

Fig. 1. The sedation-anaesthesia continuum (from Ross and Ross, 2008).

Local anaesthesia can be defined as a technique to produce reversible loss of sensation in a discrete region of the body without affecting consciousness. Local anaesthetics produce their effect by blocking propagation of action potentials in receptors and nerves at, and near to, the selected site, effectively preventing transmission of any painful stimulus to the central nervous system and blocking muscle action.

Analgesia is the relief from pain. It is a block of pain perception with or without the retention of other sensory abilities and with or without continued control of motor function. It typically does not result in loss of equilibrium. In animals it is reflected by a reduced response to noxious stimuli.

General anaesthesia may be defined as 'a reversible, generalised loss of sensory perception accompanied by a sleeplike state induced by drugs or by physical means (Heavner, 1981). It involves a state of general depression of the central nervous system relating hypnosis, analgesia, suppression of reflex activity and relaxation of voluntary

muscle' (Green, 1979) through an action on nerve axons, transmitter release or membrane excitability or a combination of these actions.

The only unifying principle of general anaesthesia is that anaesthetics interact with membrane components and no single cellular mechanism is able to explain their widespread effects in the central nervous system (Winlow et al., 1992) although they probably all modify the activity of ion channels (Arhem et al., 2003).

General and local anaesthesia can be produced by a variety of techniques including physical, chemical and psychological methods, which are summarised in **Table 1**.

Table 1. Methods used to produce general or local anaesthesia (from Ross and Ross, 2008).

Methods	Tecnique
Physical	Hypothermia
	Electrical stimulation
	Acupunture
	Pressure
Chemical	Inhalation
	Parenteral: intravenous
	intraperitoneal
	intramuscular
	Oral (enteral)
	Rectal
Psychological	*Suggestion/Distraction*
	Hypnosis

Classically, there are four recognized stages of anaesthesia:

Stage I – Analgesia (initial analgesia without amnesia)

Stage II – Excitement

Stage III – Surgical anaesthesia

Stage IV – Medullary depression (overdose)

For safe anaesthetic procedures, it is important to evaluate the depth of anaesthesia. To better evaluate the depth of anaesthesia, **stage III** has been subdivided into four planes:

Plane 1- Light anaesthesia- Regular respiratory rhythm and palpebral reflexes are still present.

Plane 2- Medium anaesthesia- Eyeball movements cease and palpebral reflexes are lost. Overall muscle tone decreases. An increase in blood pressure, heart rate, and respiration can occur in response to surgical stimulation.

Plane 3- Deep anaesthesia - Respiratory muscle paralysis begins and overall muscle tone is very relaxed. Pupillary light reflex may be slow or absent. Most surgical procedures can be done in late Plane 2 or early Plane 3.

Plane 4 -Too Deep- All muscles, including diaphragm and intercostal muscles, are paralyzed. Cardiovascular compensating reflexes are markedly diminished. Cessation of respiration with paralysis of the diaphragm signifies the end of Plane 4.

Stage IV is the period between cessation of respiration and circulatory collapse. This stage should be avoided as death is usually imminent.

1.2 General anaesthesia in fish

The use of anaesthetics is required for manipulation of animals during procedures such as transport, grading or vaccination, to reduce the stress associated with the procedures, and for invasive studies, such as surgical preparations and physiological investigations, during which the fish must be held immobile for extended periods of time. Overdose of anesthetics is also used as an effective and humane way to euthanize fish (Canadian Council on Animal Care).

The progressive stages of fish anaesthesia were first adapted and described by McFarland (1959). They include four stages:

stage I: the animal is responsive to stimuli but motion reduced, ventilation decreased (plane 1, light sedation); some analgesia, only receptive to gross stimulation (plane 2, deep sedation);

stage II: Partial loss of equilibrium; good analgesia (plane 1, light sedation); total loss of muscle tone, total loss of equilibrium, ventilation almost absent (plane 2, deep sedation);

stage III: total loss of reaction to even massive stimulation (surgical anaesthesia);

stage IV: Ventilation ceases, cardiac arrest, eventual death; overdose (medullary collapse).

Immobility of the fish is achieved by Stage III for most anesthetics; however, some anesthetics (e.g., 2-phenoxyethanol, metomidate, quinaldine sulfate) may not completely block involuntary muscle movements and muscle twitching may still occur. Such side effects may make the anaesthetic unsuitable for use if blood sampling or surgery is required.

Many of the anesthetics used on fish are similar to those used in mammals or even on humans. However, some of these are considered topical anaesthetics in mammals, whereas they are applied in a general manner to fish inducing a progressive depression of both the central and peripheral nervous system activities (Summerfelt and Smith, 1990).

1.2.1 Widely used drugs for anaesthesia in fish

Early methods of fish anaesthesia include "a blow to the head from which, with luck, the animal might later recover," electrical stunning (Healey, 1964). Later, general anaesthetics such as ether and chloroform were used, as well as a number of other chemicals such as urethane, chloretone, chloral hydrate and cocaine. Many of these have since been replaced both for their toxicity (chloretone: Malmstrom et al., 1993), and for their carcinogenic nature (chloroform and urethane: Ball and Cowen, 1959). Ether was also quite popular but its use was decreased due to its flammable nature. Chilling using crushed ice has been used (Parker, 1939) but it causes stress and the anaesthesia achieved is not very deep (Healey, 1964; McFarland, 1960). More recently it has been used as a supplement to chemical anaesthesia (Williamson and Roberts, 1981).

Today, the widely used drugs for fish anaesthesia are: MS-222, Benzocaine, Clove oil, AQUI-S Quinaldine, quinaldine sulphate, 2-Phenoxyethanol and Metomidate.

MS-222

It became popular in the 1960 and has remained popular probably because it is the only fish anaesthetic registered by the American Food and Drug Administration (FDA). MS-222 is usually administered by inhalation, but has been reported its successful application directly to the gill of adult lake trout (*Salvelinus namaycush*) using a spray (Kidd and Banks, 1990). It shows rapid induction (15 seconds) and rapid recovery times (Ross and Ross, 2008). However, a list of side effects has been documented for MS222, including elevated haematocrit, erythrocyte swelling, hypoxia, hypercapnia, hyperglycaemia, changes in blood electrolytes, hormones, cholesterol, urea, lactate and inter-renal ascorbic acid.

MS-222 and Benzocaine are effective at doses between 25 and 50 mg L−1 and have a good margin of safety. In addition, benzocaine appears to induce immunodepression in *Sparus auratus* (Ortuno et al. 2002) and has minor and insignificant effect on feed intake and growth in Atlantic salmon (Sorum and Damsgard, 2004).

Clove oil

Clove oil is distilled from the flowers, stalks and leaves of *Eugenia aromaticum* or *Eugenia caryophyllata*. The active ingredient is the oil eugenol, but raw clove oil also contains acetyl eugenol and a very wide range of turpenoid compounds, which impart their characteristic odour and flavour.

A great interest in use of clove oil as anaesthetic in fish due to its safety and effectiveness (Javahery et al., 2012). Several authors found to be effective in different fish species (*Carassius carassius*: Endo et al., 1972; *Cyprinus carpio*: Hisake et al., 1986; *Siganus lineatus*: Soto, 1995). The fish lost equilibrium after 30–45 seconds and recovery required about 3 minutes. However, it was demonstrated that clove oil reduced feed intake (Pirhonen and Schreck, 2003) and negatively affect the growth of the animals (Hoskonen and Pirhonen, 2006).

AQUI-S

The active ingredient of the product is isoeugenol, which, although very similar to eugenol, is not present in natural clove oil. Usually, exposure to this anaesthetic agent does not induce adverse reactions. Small and Chatakondi (2005) found that it was

effective as MS-222, has similar induction times and slightly longer recovery times than MS222 and showed some stress-reducing properties; however Davidson et al., 2000 examining cortisol levels and other blood parameters in AQUI-S-anaesthetised rainbow trout, affirmed that AQUI-S did not alleviate stress. Wagner et al. (2002) found that recovery time following AQUI-S anaesthesia of rainbow trout was about twice that required following MS-222 anaesthesia.

Quinaldine and quinaldine sulphate

These agents induced anaesthesia in 1–4 minutes showing a rapid and uneventful recovery. However these agents were unsuitable for maintenance during surgery at 29 °C in the grass carp, *Ctenopharyngodon idella* (Schramm and Black, 1984). In addition, Schoettger and Steucke (1970) noted during surgical anaesthesia in rainbow trout that some reflex responsiveness was retained.

2-Phenoxyethanol

Doses 385 mgL^{-1} produce surgical anaesthesia in rainbow trout, *Oncorhynchus mykiss*, and sedation can be produced at a lower dose although analgesia is sometimes incomplete. Takashima *et al*. (1983) showed a rapid increase of cortisol levels after immersion of the fish in this anaesthetic indicating no stress alleviation.

Metomidate

This drugs produce anaesthesia without elevation of blood cortisol (Olsen *et al*., 1995). However, Kreiberg (1992) noted the relatively small increase in plasma cortisol in Pacific salmon on handling following metomidate anaesthesia. It has been shown to be very effective, induction is achieved in 1–2 minutes with no hyperactivity, the recovery is faster than after MS-222 anaesthesia although it can be accompanied by muscle twitching. By contrast with these positive indications, Massee *et al*. (1995) observed high mortality.

1.2.2 Cardiovascular effects of the most common anaesthetic agents in fish

Although anaesthetic agents are widely used in fish, few studies regarding their effects on the cardiovascular performance are available. MS-222, metomidate, and AQUI-S have

been examined with respect to changes in heart rate, cardiac output, dorsal aortic pressure and stroke volume (Hill and Forster, 2004). The authors reported that during induction, MS-222 caused a decline in dorsal aortic pressure only, while metomidate did not affect any cardiovascular variable. AQUI-S depressed heart rate, cardiac output, dorsal aortic pressure and stroke volume (Hill and Forster, 2004).

2-Phenoxyethanol caused marked reduction in aortic blood pressure in rainbow trout (Fredricks *et al.*, 1993). Metomidate has been found to cause drastic reduction in dorsal aortic blood pressure in rainbow trout (Fredricks et al., 1993).

1.3 General anaesthesia in cephalopods

On the 22nd September 2010 the Directive on the "Protection of animals used for scientific purposes" (Directive 2010/63/EU) was adopted by the European Union and has been implemented by the 28 EU Member States by 1st January 2013. The new law, which replaces EU Directive 86/609, includes for the first time the use of an entire class of Molluscs, i.e. ‘‘live cephalopods’’ (larval and adults forms).

The choice to include cephalopods was based primarily upon the recommendations of a scientific panel which concluded that there was ‘‘scientific evidence of their ability to experience pain, suffering, distress and lasting harm’’. This was supported by recent neurophysiological afferent recording studies (Alupay et al., 2013; Crook et al., 2011; Crook et al., 2013), that provided direct evidence for presence of mechano-nociceptors in cephalopods.

Cephalopod are cognate far beyond all other invertebrates. Their anatomy and physiology exhibit many similarities to the Vertebrata in terms of complexity. Possessing large brains, cephalopods have extremely flexible behaviour and highly developed attention and memory capacities resembling those of some vertebrates, including discrimination and generalization, social learning and spatial awareness.

According to the new Directive, all the procedures, carried out on cephalopods, exceeding the threshold for induction of pain, suffering, distress or lasting harm (PSDLH) should be regulated in an identical way to any other classic vertebrate laboratory animals (Smith et al. 2013; Andrews et al. 2013; Fiorito et al. 2014).

In contrast to vertebrates, for the cephalopods there is a relatively rudimentary knowledge for the different issues covered by the new legislation such as: *i.* capture, transport and handling; *ii.* environmental characteristics and design of facilities (e.g. water quality control, lighting requirements, vibration/noise sensitivity); *iii.* housing, maintenance, care conditions, feeding and environmental enrichment; *iv.* assessment of health and welfare (e.g. monitoring biomarkers, physical and behavioural signs); *v.* classification of the severity of procedures; *vi.* disease (causes, prevention and treatment); *vii.* scientific procedures, general anaesthesia and analgesia, methods of humane killing and confirmation of death.

Thus, the challenges provided by the new Directive increased, in the cephalopod community, the attention to cephalopod welfare in order to indicate adequate and standardized procedures for treatment, care, and management of these animals.

For this reason, the objective of the Directive is to promote the research in the above areas to facilitate the development of specific guidelines for optimal care and welfare (Moltschaniwskyj et al. 2007;Louhimies 2011; Goncalves et al. 2012; Sykes et al. 2012; Andrews et al. 2013; Smith et al. 2013; Fiorito et al., 2015).

The new EU Directive 2010/63/EU requires that surgical or investigative procedures during scientific procedures 'are carried out under general or local anaesthesia' unless anaesthesia is judged to be 'more traumatic to the animal than the procedure itself' and/or 'is incompatible with the purpose of the procedure (Article 14.2). Member States shall ensure that, procedures are carried out under general or local anaesthesia, and that analgesia or another appropriate method is used to ensure that pain, suffering and distress are kept to a minimum (Article 14.1).

It may be necessary to anaesthetise an animal for a number of reasons including:

i. performing a minor or major experimental surgical procedure, e.g. to lesion part of the nervous system surgically (e.g. Andrews and Tansey, 1983), chemically or electrolytically (e.g. Graindorge et al., 2008);

ii. implantation of catheters (e.g. Andrews and Tansey, 1981b) or recording devices (e.g.: Brown et al., 2006; Zullo et al., 2009);

iii. to treat a veterinary problem (e.g. repair of damaged skin, Harms et al., 2006);

iv. to facilitate handling for investigation such as repeated ultrasound measurement of brain size (e.g. Grimaldi et al., 2007);
v. for transport (e.g.: Garcìa-Franco, 1992; Sen and Tanrikul, 2009);

A general anaesthetic agent should, at an appropriate dose, render an animal into a reversible state of unconsciousness and insensibility, particularly to painful stimuli. This state should last for at least the duration of the period when a potentially painful or distressing procedure, such as surgery, is performed. The agent should be non-aversive, or minimally aversive at the appropriate dose rate and there should also be no memory of the procedure (amnesia). As some substances have only a neuromuscular blocking effect (muscle relaxation or paralysis), they may produce an apparent state of general anaesthesia (no response to external stimuli) maintaining, however, the ability to feel pain and distress. Thus, the new Directive prevents the use of neuromuscular blocking agents without appropriate anaesthesia or analgesia (Article 14.2b).
Moreover, under anaesthesia, physiological functions should be maintained as closely as possible within normal ranges and since recovery from the anaesthetic is required, it is essential that there are no residual deleterious effects.

The process of anaesthesia can be divided into three basic phases: induction, maintenance and recovery. Each of these varies in duration according to drug or method used, species and conditions.

1.3.1 Induction

The induction should be a rapid process designed to reduce stress and trauma to the animals so as to safeguard animal welfare (Andrews et al., 2013). During induction the animal is exposed to the anaesthetic agent in order to achieve the desired stage. Induction is often accompanied by hyperactivity, usually a response of only a few seconds to the sensation or slightly irritant properties of the drug.
The usual practice is to take out the cephalopod from the home tank, and put it directly in a the anaesthetic solution. According to the new guidelines for the care and welfare of cephalopods (Fiorito et al., 2015), this procedure might be refinedin order to reduce adverse effects on the animals. It may be less traumatic avoid removing the animals from

the water and carry out the transfer to the anaesthetic chamber within a sea water filled tank (as in Walker et al., 1970).It is preferable to expose the animal to increasing concentrations of the anaesthetic agent to quickly identify any adverse reaction (Yacob et al., 2011).

Adverse effects may be reduced exposing the animal to oxygenated sea water (rather than directly anaesthetic), allowing them to acclimatize, and then gradually replacing the sea water with the anaesthetic solution (Andrews et al., 2013).

The animal should remain completely immersed in the anaesthetic solution for rapid effect in a closed anaesthetic chamber to prevent octopus escaping. The chamber could also be used as a transport box from the home tank to the operating room, and it may be possible to habituate at least some species (e.g. octopus and cuttlefish) to the box and train them to enter. Generally, animals should always be transported in seawater and movement should be minimised.

Anaesthetic solutions should always be freshly made, using filtered seawater, which is gassed and equilibrated to home tank temperature before immersing the animal.

It is not good practice to anaesthetize an animal in a solution that has been used to anaesthetize another animal, as the water may contain chemical alarm signals. In addition, animals must not be immersed in an anaesthetic solution in which they have inked.

1.3.2 Maintenance

Maintenance involves extending the achieved stage in a stable manner without detriment to the health of the animal. It should be uneventful and effective on a reduced drug dose. When animals achieved anaesthetized state (see criteria to asses general anaesthesia), it is needed to remove it from the anaesthetic chamber to perform a procedure. Anaesthesia must be maintained for the entire duration of the procedure, and physiological functions monitored.

Marked suppression or cessation of ventilation (indicated by mantle/siphon contraction) is a common feature of general anaesthesia in cephalopods, so the mantle perfusion with oxygenated seawater is necessary . Few information are available about cardiac function under anaesthesia; it has been observed that heart rate is very lowin *O. vulgaris*

anaesthetised (M.G. Valentinoand P.L.R. Andrews, unpublished observations) following exposure to $MgCl_2$ and cold water.

Since the physiological functions may impact on post-operative recovery and procedures the monitoring of physiological function under anaesthesiaand during surgery is clearly an area requiring research.

Doppler ultrasound (e.g. as in D. Fuchs and G. Ponte, unpublished observations; Vevo 2100Visualsonics, The Netherlands) offers the best techniquefor monitoring cardiovascular function.

1.3.3 Recovery

The recovery phase involves withdrawal of the animal from the anaesthetic agent and his return to a normal state. Initial recovery may take anything from a few seconds to a few minutes, but in general it should be quick and without altered behaviour or other side-effects.

During recovery the animal should be placed in clean aerated/oxygenated seawater. Ventilation can often start without intervention (depending upon the duration of anaesthesia), but in cuttlefish, squid and octopods a gentle massage of the mantle is frequently used until ventilation restart.

Other functions(sucker adhesion, chromatophore tone, righting) recover after ventilation recommences, usually in the reverse order to which they were lost.

As currentlyassessed, recovery appears rapid (less than 15 mins, e.g. Gonçalves et al., 2012) and dependent on the procedure(s) performed, animals will usually take food quickly after ‘anaesthesia’ when returned to their home tank. (Agnisola et al., 1996).

However, further monitoring criteria are needed toensure that animals have fully recovered from the anaesthesia and any surgical procedure.

1.4 Anaesthetics for cephalopods

Based on the literature data regarding anaesthesia in cephalopods, few information on which agents - and in which species- provide the most effective general anaesthesia (i.e. block of nociception and pain perception, no aversion, rapid induction and fast recovery without adverse effects) is available (Andrews et al., 2013; Fiorito et al., 2015).

The potential anaesthetic proprieties of about twenty agents or combinations have been investigated on cephalopods (reviewed in Gonçalves et al., 2012; Andrews et al., 2013; Gleadall, 2013; Fiorito et al., 2015). The following agents have been utilized: benzocaine (ethyl p-amino-benzoate); carbon dioxide; chloretone; chloroform; chloral hydrate; cold sea water; clove oil; ethanol; magnesium chloride; magnesium sulphate; menthol; metomidate; MS222 (ethyl-m-aminobenzoate, also known as tricaine mesylate, tricaine methylsulphonate, Metacaine, Finquel); nicotine sulphate; 2-phenoxyethanol (phenoxetol); propoxate and urethane (ethyl carbamate).

Species in which anaesthetic agents have been investigated include: *Allotethis subulata, Amphioctopus fangsiao, Argonauta argo, Doryteuthis pealei, Eledone cirrhosa, Eledone moschata, Enteroctopus dofleini, Idiosepius notoides, Loligo forbesi, Loligo vulgaris, Nautilus pompilius, Octopus vulgaris, Octopus tetricus, Pinnoctopus variabilis, Sepia officinalis, Sepioteuthis lessoniana, Sepioteuthis sepioidea and Watasenia scintillans.*

The majority of the anaesthetics used so far in cephalopods did not show satisfactory effects either to their toxic effects (even at low concentrations) or to their ineffectiveness (**Table 2**).

Low doses of chloretone and gallamine produced no anaesthetic effects or fatalities (in *Octopus vulgaris* and *Amphioctopus fangsiao* respectively***)***, but trials with higher doses and/or longer exposure caused fatalities without an anaesthetic state being reached (Gleadall, 2013).

In *Octopus vulgaris*, nicotine sulphate was fatal whatever the exposure time, although some anaesthetic effect occurred. However, before induction the octopuses showed symptoms of discomfort, were not anaesthetised fully and did not recover (Gleadall, 2013).

Phenoxetol (2-PhOH) was demonstrated to be an effective and safe anaesthetic for the musky octopus, *Eledone moschata* at doses between 1.2 and 1.6 mL L^{-1} that did not cause any mortality or toxicity in the musky octopus during 48h (Sen and Tanrikul, 2009). On the contrary, Gleadall showed that phenoxetol was always fatal for *Octopus vulgaris* and *Eledone moschata* whatever the exposure time, although some anaesthetic effect occurred. In addition, in *Sepia officinalis*, phenoxetol did not produce suitable values of

induction or recovery times and behaviour reactions of animals were too severe with mortality in most trials (Gonçalves et al., 2012).

Metomidate was toxic even at low concentrations and evoked a severe reaction from octopuses,; quinaldine sulphate induced copious mucus production by *Amphioctopus fangsiao* placed in the substance (Gleadall, 2013).

MS-222 produced no useful anaesthetic effect and severe reactions from the octopuses (*Octopus vulgaris* and *Enteroctopus dofleini*: Gleadall, 2013; *Sepia officinalis*: Gonçalves et al., 2012). Similar results were observed with propoxate in *Amphioctopus fangsiao* and *Octopus vulgaris.*

Higher concentrations or longer exposures to chloretone, MS-222, metomidate, nicotine sulphate and propoxate commonly induced a set of effects termed 'typical adverse response pattern' (TARP). These effects are characterized by frequent escape attempts initially, and an increase in breathing rate to almost double that at rest. Breathing then becomes erratic, eventually slowing. Basic body muscle tone and posture, 'sticky' suckers, tactile and visual reflexes are all retained throughout, although visual awareness may be somewhat dulled. Also typical is a hypersensitive response to tactile stimuli, even from a very gentle knock on the laboratory bench. There are frequent rapid colour changes between pale and dark; spontaneous deimatic displays; flashing of chromatophores on the body and on the base of the arms (not usually seen on the arms themselves); exaggerated raising of papillae, especially around the eyes and sometimes on the dorsal mantle; sporadic jerking movements (sometimes including sudden rotations of up to 20°), sudden widening of pupils, convulsions, defaecation and copious inking. On some occasions, the mantle takes on a strange where the posterior 20–25% of the mantle remains unusually constricted while the main part of the mantle musculature continues the typical cycle of expanding and contracting movements associated with irrigation of the gills during breathing.

Clove oil appears to have sedative/anaesthetic properties in *Octopus minor* (Seol et al., 2007) and*Sepia elongata* (added to ethanol; Darmaillacq and Shashar, 2008). It induces minimal immersion trauma, rapid induction, good recovery and minimal mortality.

Clove oil has been also investigated in *O. vulgaris*, although an anaesthetic concentration was not identified (Estefanell et al., 2011). The authors demostrated that clove oil was ineffective while immersion in seawater with 1.5% of ethanol (96%) showed a rapid anaesthetic time without the drastic effects shown by the higher concentration. So

further studies are required to assess its potential as an anaesthetic agent in species of octopus, either a single agent or as an adjuvant to magnesium chloride.

Furthermore, in *Dorytheuthis pealei* Mooney et al. (2010) reported jetting, inking, chromatophore flashing and death in four minutes at higher doses of clove oil, suggesting that responses are both dose- and species-specific. Additional studies are therefore required to assess the suitability and appropriate doses of clove oil for different species of cephalopod.

Urethane has been used successfully in cephalopods (Andrews and Tansey, 1981; Messenger et al., 1985; Young, 1971a), but it is now discarded because ofits carcinogenic properties, including leukopenia and chromosomal damage in humans (Wood, 1956; Ball and Cowen, 1959; Mirvish, 1968) as well as welfare reasons. Gleadall (2013) reported that, even if, octopus showed no signs of stress during induction, the full anaesthetic state was notachieved because occasional arm and breathing movements resumed sporadically.

Table 2. Summary of effect of anaesthetic substances (or hypothermia) tested on octopuses to date.

ANESTHETIC	SPECIES	CONCENTRATION	TIME OF INDUCTION (MIN)	TIME OF RECOVERY (MIN)	EFFECTS	REFERENCES
BENZOCAINE	*Doryteuthis pealeii*	0.28 g L^{-1}	-	-	RAF	Mooney et al., 2010
CHLORETONE	*Octopus vulgaris*	0.01 g L^{-1}	-	-	NAA, FR	Gleadall, 2013
	"	0.5 g L^{-1}	-	-	TARP, DD	"
	"	1 g L^{-1}	1.5, 5.5	23, 60	TARP, DD	"
CLOVEOIL	*Octopus minor*	0.05 – 0.3 g L^{-1}	14-4 (20°C)	13-24 (20°C)	GREAT	Seol et al., 2007
	Octopus vulgaris	0.002 – 0.01 g L^{-1}	-	-	NAA	Estefanell et al., 2011
	Doryteuthis pealei	1 ml L^{-1}	-	-	RAF	Mooney et al., 2010
	Sepia officinalis	0.05, 0.15 ml L^{-1}	-	>15	Stressful reactions	Gonçalves et al., 2012
ETHANOL	*Amphioctopus fangsiao*	5 ml L^{-1}	20	<1	SaA, FR	Gleadall 2013
	Amphioctopus fangsiao	10 ml L^{-1}	2, 20	16, 2.5	Bmm not abolished, FR	"
	Octopus vulgaris	10 ml L^{-1}	7-8	3-7	SA, FR	"
	"	20 ml L^{-1}	1.5-10	5	SaA, FR	"
	"	30 ml L^{-1}	6	-	SaA	"
	Enteroctopus dofleini	30 ml L^{-1}	10-15	10	II, FR	"
	Octopus vulgaris	10, 15, 20 ml L^{-1}	4-0.8	3-6	GREAT (at 15 ml L^{-1})	Estefanell et al., 2011
	"	20 ml L^{-1}	-	-	Successful with IJ	Andrews &Tansey, 1981
	"	20 ml L^{-1}	15-40	-	IJ, SA or SaA (*)	Pagano et al., 2011
	Doryteuthis pealeii	10 ml L^{-1}	-	-	GREAT with IJ, Ac	Mooney et al., 2010
		30 ml L^{-1}	4	-		
	Sepia officinalis	10 ml L^{-1}	7.2	1.1	NO Stressful reactions	Gonçalves et al., 2012
		20 ml L^{-1}	1.5	1.5	NO Stressful reactions	"
		30 ml L^{-1}	1.2	1.7	Stressful reactions	"

ANESTHETIC	SPECIES	CONCENTRATION	TIME OF INDUCTION (MIN)	TIME OF RECOVERY (MIN)	EFFECTS	REFERENCES
HYPOTHERMIA	*Amphioctopus fangsiao* *Octopus vulgaris* *Doryteuthis pealeii* *Sepia officinalis*	4°C “ “ 8°C	2-3 - - 1.8	2 - - 4.2	NAA, IJ, EA, II LTA LTA Stressful reactions	Gleadall, 2013 Andrews & Tansey, 1981 Mooney et al., 2010 Gonçalves et al., 2012
ISOFLURANE	*Octopus vulgaris*	0.5-2.5 %	5 (2%)	45-60 (2%)	GREAT	Polese et al., 2014
MAGNESIUM CHLORIDE	*Doryteuthis pealei* *Amphioctopus fangsiao* *Octopus vulgaris* *Octopus vulgaris* *Sepia officinalis*	30.5 g L-1 32.5 g L-1 35 g L-1 35 g L-1 20 g L-1 27 g L-1	3.1 2-8 10-15 20 7.8 6.1	- 11 - 5 6.4 5.9	GREAT “ “ “ GREAT “	Mooney et al., 2010 Gleadall, 2013 Grimaldi et al., 2007 Pagano et al., 2011 Gonçalves et al., 2012 “
MAGNESIUM CHLORIDE /ETHANOL	*Octopus vulgaris* *Octopus vulgaris* *Octopus vulgaris,* *Sepia officinalis*	11.2 g L-1 $MgCl_2$ / 10 ml L-1 EtOH 11.2 g L-1 $MgCl_2$ / 10 ml L-1 EtOH 11.2 g L-1 $MgCl_2$ / 10 ml L-1 EtOH	0 - 25-45 10-20	>50 - - -	GREAT GREAT GREAT	Pagano et al., 2011 Shomrat et al., 2008, Shomrat et al., 2011
MAGNESIUM CHLORIDE (SW:dH2O)	*Octopus vulgaris* *Sepia officinalis* *Loligo forbesi* *Alloteuthis subulata* *Octopus vulgaris* *Eledone cirrhosa*	35 g L-1 (SW: dW) (1:1) 32.5 g L-1 (SW: dW) (1:1)	26 -	8 -	GREAT “	Pagano et al., 2011 Messenger et al., 1985

ANESTHETIC	SPECIES	CONCENTRATION	TIME OF INDUCTION (MIN)	TIME OF RECOVERY (MIN)	EFFECTS	REFERENCES
METOMIDATE	*Amphioctopus fangsiao*	0.01-0.15 g L-1	-	-	NAA	Gleadall, 2013
MS-222	*Octopus vulgaris* “ “ “ *Enteroctopus dofleini* *Octopus vulgaris* “ “	0.005 g L-1 0.02 g L-1 0.04 g L-1 0.05 g L-1 0.1 g L-1 < 0.03 g L-1 >0.03 g L-1	17 43 54 12 40 - -	- >30 4 5 - - -	SA, FR II II, Co TARP Vcm, IJ NAA Co, IJ	Gleadall, 2013 “ “ “ “ Pagano et al., 2011 “
NICOTINE SULPHATE	*Octopus vulgaris*	25 ml L-1 50 ml L-11	14 14	- -	TARP, RRA, DD “	Gleadall, 2013 “
PHENOXYETOL	*Octopus vulgaris* “ *Eledone moschata*	0,05 ml L-1 0,5 ml L-1 1-1.8 ml L-1	19 9 10.5-8.8	- - 12.5- DD	IJ, DD or FR IJ, DD NAA, RAF	Gleadall 2013 “ Sen &Tanrikul, 2009

ANESTHETIC	SPECIES	CONCENTRATION	TIME OF INDUCTION (MIN)	TIME OF RECOVERY (MIN)	EFFECTS	REFERENCES
PROPOXATE	*Octopus vulgaris*	2.5*10-4 g L-1	43	>26	EA, II, FR	Gleadall, 2013
	"	0.001 g L-1	-	-	EA, II, FR	"
	"	0.002 g L-1	-	-	NAA, FR	"
	"	0.006 g L-1	46	~50	TARP, II, FR	"
	Amphioctopus fangsiao	0.003 g L-1	-	-	Ia, TARP, Be, FR	Gleadall, 2013
	"	0.004 g L-1	-	-	Ia, TARP, FR	"
	"	0.005 g L-1	-	10	Ia, TARP, FR	"
	"	0.006 g L-1	27	-	TARP, DD	"
	"	0.01 g L-1	3	-	TARP, II, DD, FR	"
	"	0.029 g L^{-1}	5	-	II, FR or DD (*)	"
URETHANE	*Amphioctopus fangsiao*	30 g L-1	25	-	SA	Gleadall, 2013
	Octopus vulgaris	30 g L-1	-	-	IJ	Andrews & Tansey, 1981

Abbreviations: Ac, Attached to container; Be, Breathing erratic; Bmm not abolished, Breathing, muscular movements not abolished; Co, convulsion; DD, Died; EA, Escape Attempts; FR, Full Recovery; GREAT, good results without adverse effects; Ia, Incomplete anaesthesia; II, Incomplete Induction; IJ, Ink Jetting; LTA, Lack True Anesthesia; NAA, No Anesthetic effects Apparent; RAF, Reaction Adverse, usually fatal; TARP, Tipical Adverse Response Pattern; RRA, Righting Reflex Abolished; SA, Shallow Anesthesia; SaA, Satisfactory Anesthesia; Vcm, Violent contractions of mantle; *, exposure-dependent effects.
(Modified from table 3 of Gleadall, Journal of Experimental Marine Biology and Ecology, 2013).

Among this extensive list of anaesthetic procedures used in cephalopods, we focused on the most common ones utilized in *Octopus vulgaris* for both their effectiveness and minimal adverse effects.

1.4.1 Hypothermia

Hypothermia was widely used by the cephalopod research community. An advantage of hypothermia ('cold narcosis' *sensu* McFarland and Klontz, 1969), is avoidance of drugs and their potentially toxic effects. It was first used on *O. vulgaris* by Andrews and Tansey (1981), who pointed out that this drug-free procedure offers a stable and suitable preparation for neuropharmacological experiments. However, due to the rigid conditions of refrigerated animals, surgery that requires incision of the mantle muscle was more difficult (Andrews and Tansey, 1981).

Hypotermia was also investigated on *Sepia officinalis* by Goncalves et al., (2012) demonstrating that this anesthetic method presented induction times (110.00±52.01 s) similar to ethanol doses (20 and 30 mL/L^{-1}) and recovery times similar to other anaesthetics (clove oil and AQUI-S). However, hypothermia recovery times (254.80±167.68 s) were very irregular, presenting a high value of standard deviation. Thus, despite no mortality occurred, this agent has not been recommend in cuttlefish for a short-term handling, being the behavior of cuttlefish harsh, with animals showing severe stress reactions during the induction and recovery stages. These Authors did not define hypothermia as an anaesthetic agent but a way of reducing cephalopod metabolism to lower levels, so the animal will probably still be sensible whatever procedure that is being performed (Goncalves et al., 2012).

Cold seawater containing 2% ethanol has been also used to deeply anaesthetize *O. vulgaris* prior to brain removal (Hochner et al., 2003) but studies where this and other combinations with cold sea water have been used for humane killing are not available (Andrews et al., 2013).

1.4.2 Ethanol

A large number of studies have been performed by using ethanol (EtOH), as anesthetic agent for cephalopods, (Gleadall, 2013 and references therein). In the range 1-3%, EtOH induced a rapid induction of full anaesthesia, adequate duration, swift recovery, and no

adverse reactions, even if it was observed an inadequate (incomplete) induction at lower temperatures (below 10 °C). This latter effect has been explained by the decline of EtOH narcotising effects at lower temperatures (Moore et al., 1964).

In contrast, good results with ethanol at cold temperatures have been reported for *E. dofleini* at 10–13 °C (Harrison and Martin, 1965) and *Eledone* at 9–18 °C (Boyle, 1981).

EtOH (1-3%) effectively anesthetized the squid *Doryteuthis pealeii* for periods up to 74 min, but induced adverse reaction indicated by muscle tension, specifically that of attaching their suckers to the side or bottom of the bin, jetting and repeated dramatic color/body pattern changes, from a deep, rust-colored red to pale (Mooney et al., 2010).

Ethanol (2%) is also considered effective in *O. vulgaris*, although aversive effects, based on escape attempts and ink release, have been reported (Andrews and Tansey, 1981; Froesch and Marthy, 1975).

1.4.3 Magnesium chloride

In various formulations, $MgCl_2$ has been used to anaesthetize species representative of the three major orders of cephalopods, Sepiida, Teuthida and Octopoda, i.e.: *Sepia officinalis, Loligo forbesi, Dorytheutis pealei, Sepioteuthis sepioidea, Ilex illecebrosus, Octopus vulgaris,* and *Eledone cirrhosa* (Messenger et al., 1985; Pörtner at al., 1991; Garcia-Franco et al., 1992; Mooney et al., 2010; Gleadall, 2013b; Wearmouth et al., 2013).

In comparison to other agents, no adverse effects and very low post-anaesthetic mortality were observed (Messenger et al., 1985; Mooney et al., 2010). Induction of the full anaesthetic state was rapid, within a few minutes, duration was typically 10–20 min, recovery was roughly as swift as induction. In some instances it has been also combined with ethanol as, for example, in *S. officinalis* (Graindorge et al., 2008) or *O. vulgaris* (Shomrat et al., 2008). Shomrat and coworkers (2008) used a combination of 55mM $MgCl_2$ (approx. 0.5% w/v) and 1% ethanol which induced 'deep anaesthesia' after around 25 to 45 minutes, with rapid recovery, and critically did not block the formation of Long Term Potentiation (LTP) in the vertical lobe of the brain.

Magnesium chloride (either in sea water or as mixture of sea water and distilled water) fulfils the criteria commonly used for assessing general anaesthesia in cephalopods: skin pallor (mantle and arms) and loss of texture, loss of arm muscle tone and sucker adhesiveness, loss of the righting reflex, absence of a response to a noxious mechanical

stimulus applied to the arms or mantle and marked suppression of ventilation (Andrews and Tansey, 1981a; Andrews et al., 2013; Gleadall, 2013b; Fiorito et al., 2015).

1.5 Criteria for assessing depth of anaesthesia in cephalopods

The assessment of depth of anaesthesia and physiological status of animals under general anaesthesia is pivotal to identify adequate anaesthetic protocols for cephalopods. The inclusion of cephalopods in Directive 2010/63/EU has prompted reconsideration of both criteria for general anaesthesia in cephalopods and for physiological and pharmacological effects of the common anaesthetic agents used. On the basis of a review of recent studies on cuttlefishes, squid and octopuses (Gongalves et al. 2012; Andrews et al., 2013; Fiorito et al., 2015), a detailed revision of criteria for general anaesthesia in cephalopods, is now available.

Actually, the criteria for assessing depth of anaesthesia include depression of ventilation, decrease in chromatophore tone (paling), reduced arm activity, tone and sucker adhesiveness, loss of normal posture and righting reflex, loss of response to a noxious stimulus (Andrews et al., 2013; Fiorito et al., 2015).

1.5.1 Depression of ventilation

The reaction to exposure to the anaesthetic is represented by an initial increase in depth and frequency of mantle contractions with attempts to eject the solution *via* the siphon. Subsequently, the frequency decreases progressively with time after exposure, and the coordination between the mantle and siphon become uncoordinated. With prolonged exposure, mantle and siphon contractions cease (Fiorito et al., 2015). This stimulation of ventilation may be due to an increase in activity stimulated by the chemical effects of the anaesthetic, or by the stress associated with handling, or contact with a possibly “irritant” or “unpleasant” solution (Andrews et al., 2013).

1.5.2 Decreased chromatophore tone

Paling is an early sign of “anaesthesia” and is accompanied by smoothing of the skin (i.e. loss of its 3-D texture) (Andrews et al., 2013). The overall paling of the animal is is determined primarily by the chromatophores innervation, which arises directly from the

sub-oesophageal lobes of the brain (Messenger, 2001). However, it is uncertain whether this paling is a centrally-controlled or solely peripheral effect, and so, as noted above, might not in fact indicate that the animal is unconscious. However, although animals become pale overall with increasing time of exposure to the anaesthetic, flashing colour changes have been reported on initial exposure to anaesthetic agents in *D. pealeii* (Mooney et al., 2010) or in *S. officinalis* (Gongalves et al., 2012).

1.5.3 Reduced arm activity, tone and sucker adhesiveness

The initial reaction toanaesthetic exposure may be an increase in activity (i.e. agitation, for review see Gleadall, 2013). Gradually activity decreases, including swimming activity and fin movement, as for example in cuttlefish and squid (Wearmouth et al., 2013, O'Dor et al., 1990). Octopuses will tend to settle on the bottom of the tank as the arms and suckers begin to lose tone and adhesion showing a gradual relaxation of the animals.

1.5.4 Loss of normal posture and righting response

As animals (e.g. squid and cuttlefish) become deeply 'anaesthetised' they lose the ability to maintain a normal position in the water column or adopt an abnormal position with the arms, head and mantle at angles not normally seen in conscious animals. For example, in squid and cuttlefish the arms and head may appear unsupported by the mantle collar muscles; octopuses adopt a flattened appearance on the floor of the tank rather than the usual posture with the head raised.

1.5.5 Decreased or loss of response to a noxious stimulus

General anaesthesia cannot be considered to have been achieved if pain perception is not block. Studies have used a mechanical stimulus (e.g. a pinch) applied to the arm, mantle or supraorbital skin as a test of insensibility to a noxious stimulus (Wearmouth et al., 2013; Andrews and Tansey 1981; Messenger et al., 1985). This type of evaluation is useful considering that it is known that cephalopods possess mechano-sensitive peripheral nociceptors (Alupay et al., 2013; Crook et al., 2013).

2. Cardiocirculatory system of cephalopods

2.1 Anatomy of the cardiovascular system

Among Molluscs, the cephalopods are unique in having a closed circulatory system characterized by a double circulation with three hearts, i.e. a main systemic heart and two branchial hearts (Wells, 1980). The principal pumps for the system (most of the veins show peristalsis and also help to move the blood) are the two branchial hearts, which drive the blood through the gill capillaries, and the single systemic heart into which the gills drain, supplying the rest of the body. The dual circulation, with respiratory and systemic circuits in series, reminds the system of higher vertebrates rather than fish (Wells, 1992). The systemic heart, which consists of two auricles and a single ventricle generates the high pressures necessary to push blood through an arterial system running to all major organs of the body. Three arteries leave the ventricle: the major aorta and the abdominal and gonadial arteries (Kling and Schipp, 1987a).

The branchial circulation between the veins and the systemic heart allows a full oxygenation of the blood leaving the ventricle. The blood is driven at high pressures by the heart through a complex circulatory system that is able to sustain metabolic rates almost comparable to some vertebrates (Shipp, 1987). To sustain this high oxygen uptake rates, paired branchial hearts have evolved to pump venous blood through the gills, after which the arterial blood flows to the ventricle where it is pumped to the systemic circuit. The low pressure blood returns from the body tissues *via* the anterior vena cava which divides into two lateral vena cava, reaching each branchial heart. The oxygenated blood from each gill is collected in the efferent branchial vessel from which it returns to the main systemic heart.

In addition to their function of overcome the peripheral resistance of the branchial blood vessels and capillaries, the pulsatile activity of branchial hearts is also required for the ultrafiltration processes involved in the formation of primary urine, which occur in the branchial heart appendages (pericardial gland) (Harrison and Martin, 1965; Martin and Aldrich, 1970; Potts, 1967; Schipp and Hevert, 1981).

The octopod systemic heart consists of a muscular myocardium covered by the pericardium which has no connection to a greatly reduced coelomic cavity. The epicardium lies upon a lamina basalis, connected to the myocardium through a thin layer of collagenous fibers. A layer of flattened endothelial cells form an incomplete

endocardium. The outer myocardium appears compact, with the cardiac myocytes organized in circular and longitudinal layers which appear well developed in *Eledone moschata* and *Octopus vulgaris*. In contrast, its inner surface is highly trabeculated, with muscle cells less packed towards the lumen and forming a spongy trabecular network.

The blood, which flows through the spongy myocardium from the lumen, collects in a peripheral venous system and then proceeds back to the central circulatory system through paired *'venae cordis*' (Kling and Shipp, 1987a). Having a functional analogy with the blood supply system of vertebrates, this capillary-venous system of octopods has been defined as a coronary system.

There is evidence that also in *Octopus vulgaris* a rich network of capillaries, which directly originates from the luminal trabecular spaces, supplies the myocardium. This capillary system gives rise a venous system to ensure the drainage (Agnisola et al., 1990; Agnisola, 1990).In the isolated and perfused *Octopus vulgaris* heart, theflow through this coronary system is driven by the ventricular pressure generated during the systole. It means that, the venous coronary flow is not dependent on the systemic arterial pressure, as in the vertebrate heart, but it is directly dependent on the intraventricular pressure (Agnisola, 1990; Agnisola et al., 1990; Agnisola and Houlihan 1991).

Houlihan and coworkers (1987) reported that in the isolated and perfused *Octopus vulgaris* heart, the coronary output accounted for more than 80% of the total oxygen consumption of the heart. *In vitro*, a relationship between aortic and coronary outputs of was reported also in relation to the oxygen content of the perfusate (Foti et al., 1985; Agnisola and Houlihan, 1991).

2.2 Cardiac regulation

The cephalopod cardiovascular system has a very elaborate innervations. In the Octopoda the three hearts are innervated from the sub-oesophageal part of the brain through the two visceral nerves. The visceral nerve on each side passes into a fusiform ganglion (i.e. first cardiac ganglion of some authors), and from there to a cardiac ganglion, attached to the branchial heart. A commissure links the two fusiform ganglia. In addition, there is a chain of small ganglia along the length of each gill which connect to the rest of the system through the cardiac ganglion on each side (Wells, 1980) (**Fig. 2**).

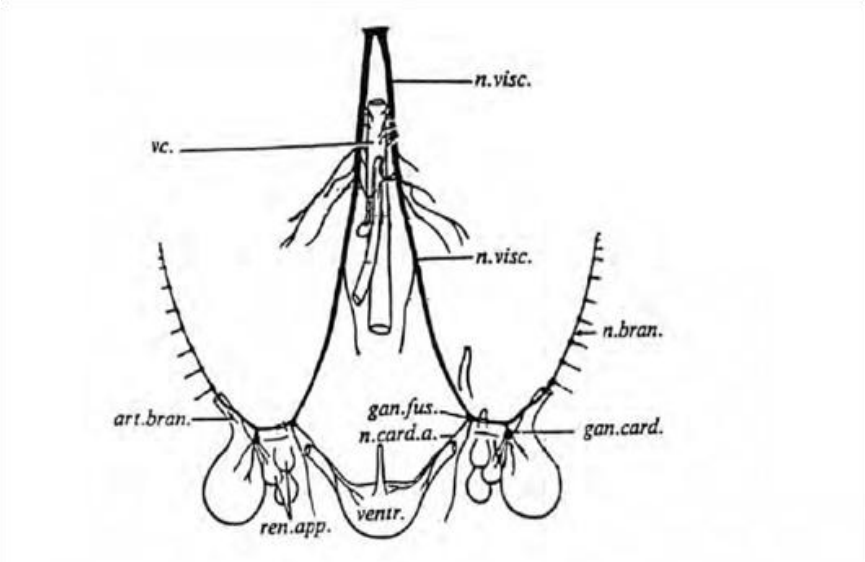

Fig. 2. The distribution of the visceral nerves in octopods [From Young (1971a)]. Artery branchialis (srt. bran.), ganglion cardiacum (gan. card.), ganglion fusiform (gan. fus.), nervus branchialis (n. bran.), nervus cardiacum atrium (n. card. a.), nervus visceralis (n. visc.), renal appendages (ren. app.), vena cava (vc) and ventricle (ventr.).

The cardiac system appears to be self-regulating, as cutting the visceral nerves and fusiform ganglia had little effect. Wells (1980) demonstrated that if both visceral nerves were cut, the heartbeat did not stop and the branchial hearts continue to beat in phase with one another as in control octopuses. The systemic heartbeat remains in phase with the branchial hearts. Heartbeat frequencies, mean aortic pressures and the aortic pulses of resting animals have similar values before and after the procedure. Removing the fusiform ganglia had no additional effects upon systolic or diastolic pressures or upon the frequency of the systemic heartbeat. In addition, as in intact octopus(Wells, 1979), the beat rate is still related to temperature and oxygen tension (Wells, 1980).

In contrast, the removal of the cardiac ganglia induced an alteration of the regular beat of the corresponding branchial heart that generally becomes disorganized. This induced a delay of the systemic heart beat that appeared irregular. However, after cardiac ganglia removal, the beat rate responds to temperature in the same way as that with the ganglion intact (Wells, 1979). This suggested that the cardiac ganglion as the likely site of the main pacemaker on each branchial heart (Wells, 1980). Since the two branchial hearts contract together, even after cutting the nerves that connect the two cardiac ganglia

through the fusiform ganglia, this implies that the two branchial heart pacemakers are normally brought into phase by events upstream.

In addition, numerous physiological and pharmacological investigations have indicated a myogenic automatism of the isolated systemic heart of different cephalopods (Klingand Schipp, 1987; Johansen and Huston, 1962; Wells and Marigold, 1980). Wells and Smith (1987) later supported the possibility of a pacemaker zone in the AV region. The ventricular myogenic automatism localized in the vicinity of A-V valves was strongly supported by other reported indications obtained trough zonation of the ventricle by ligatures (Agnisola, 1994).

2.3 Cardioregulatory substances

Several authors reported that ventricular myogenic automatism is under neurohumoral control (Kling and Jakobs, 1987; Jakobs and Schipp, 1992). The heart of closely observed cephalopods is sensitive to acetylcholine, catecholamines but also to serotonin (5HT) showing negative or positive inotropic and chronotropic effects during application of these substances.

2.3.1 Cholinergic innervations

Kling (1986) showed histochemically that acetylcholinesterase is present in the cardiac nerves and in the heart muscle itself of Sepia officinalis. Pharmacological studies demonstrated that ACh displayed a reduction of contraction and frequency in the isolated and perfused cephalopod heart (*Sepia officinalis*: Kling, 1986; Schipp et al., 1986; Kling and Jakobs, 1987; *Octopus vulgaris*: Wells and Mangold, 1980).

2.3.2 Aminergic innervations

Kling and Schipp (1987b) reported that noradrenaline causes marked increases in amplitude and frequency in the heart. In the branchial hearts, dopamine, noradrenalineand adrenaline cause an increase in amplitude but not frequency (Fiedler and Schipp, 1990). Of note, the effects of noradrenaline (and adrenaline and tyramine) differ *in vitro* from *in vivo* preparations, where they appear to be inhibitory (Wells and Mangold, 1980).

2.3.3 Indoleamines.

The effects of serotonin (5-HT) upon the systemic heart *in vitro* and *in vivo* are characterized by an increase in beat amplitude and frequency. *In vivo* injections of 5-HT induced an increase of mean pressure, pulse amplitude and beat frequency in branchial heart at lower doses, while at higher doses these effects were preceded by a temporary inhibition (Wells and Mangold, 1980).

2.3.4 Peptidergic innervations.

A peptidergic innervation seems to be present in the cardiac tissue of cephalopods (Kling and Jakobs, 1987). The authors reported that the isolated systemic heart of Sepia officinalis can be stimulated in a dose-dependent way by the 'molluscan neuropeptide' FMRFamide. In addition, Agnisola and coworkers (1989) reported that the systemic heart of *Octopus vulgaris* is a putative target of different cardiac peptides (cardiodilatin 1-16,ANF 8-33 and atriopeptins I and III). ANF, atriopeptin I and atriopeptin Ill exert both negative chronotropic and inotropic effects. On the contrary, Cardiodilatin 1-16, has very little effect on heart rate, but it exerts a potent positive inotropic effect.

3. Aims

Aim of this PhD project is to contribute to the understanding of the effects of the currently utilized 'anaesthetic' agents on the cardiovascular performance in *Octopus vulgaris*.

Although several agents have been described over the years for cephalopods, most studies have been focused to determine induction, sedation and recovery times and some behavioural description, without exploring the physiological response including cardiac function of cephalopods under anaesthesia.

Magnesium chloride ($MgCl_2$) represents the most currently used anaesthetic in cephalopods; its use covers more than 75 years (e.g. Mitolo, 1938), but the publication of a detailed study of its utility in several cephalopod species by Messenger et al. in 1985 led to a an increase in its use. The use of magnesium chloride as anaesthetic agent was also stimulated by identification of the carcinogenicity of urethane which was a widely used anaesthetic agent for surgery in octopus and other cephalopods (e.g. Boycott and Young 1955; Young 1971a; for review see also Gleadall, 2013).

According to one of the objectives of the new Directive, the specific aim of the present study was to investigate the effects of magnesium chloride on the cardiac function of *O. vulgaris* which may affect recovery from anaesthesia. The systemic heart is the focus of this study.

By using an *in vitro* isolated and perfused systemic heart, the effects of three different magnesium chloride formulations were investigated: i. 3.5% magnesium chloride made up in sea water (Margheri et al., 2011; Pagano et al., 2011; Shomrat et al., 2008); ii. 3.5% magnesium chloride made up in a 1:1 mixture of sea water: distilled water (Messenger et al., 1985; Pagano et al., 2011); iii. 1.12% magnesium chloride and 1% ethyl alcohol mixture dissolved in sea water (Shomrat et al., 2008, 2011; Pagano et al., 2011; Grimaldi et al., 2013).

The study aimed to address three questions:

1. Does exposure to $MgCl_2$ formulations *in vivo* have residual effects on the heart and, if so, could these compromise recovery from anaesthesia?
2. Does *in vivo* exposure to $MgCl_2$ formulations induce stress response in cardiac tissue?
3. What direct effects do $MgCl_2$ formulations and ethanol have on cardiac function?

4. Materials and methods

4.1 Animals

Octopus vulgaris of both sexes (males: N = 32; females: N = 18; body weight: 574 ± 25 g, mean±sem) were caught in the Bay of Naples (Italy) by local fishermen and transported to Arcavacata (Cosenza, Italy) according to the best-practice for long-duration transportation (Byrne et al., 2004; review in Fiorito et al., 2015). Octopuses were housed individually in opaque tanks (40 x 50 x 80 cm) with circulating sea water (18-22°C) and maintained according to the best practice (e.g. Agnisola et al., 1996) for up to three days prior to humane-killing. Animals were randomly assigned to the various experimental groups.

Age of octopuses cannot be estimated in live individuals, but assessed *post-mortem,* and does not appear to correlate with body weight (Canali et al., 2011). Therefore information on age of the octopuses was not possible to take into account at the time the animals were assigned to experimental groups.

4.2 Regulatory considerations

Research studies involving "live cephalopods" within the EU are covered by Directive2010/63/EU (European Parliament and Council of the European Union 2010) and itssubsequent transposition into legislation of Member States (see also: Smith et al.,2013; Fiorito et al., 2015). The studies reported here were performed before Italy transposed the Directive(March 2014). In addition, these experiments involve killing the animal for the solepurpose of removing tissue. Therefore they fall outside the scope of the Directive 2010/63/EU, provided that an approved method of killing is used (see below). Ethicalreview of the experiments at institutional level was undertaken at the study site (Cosenza, Italy). This study adhered to the ethos of the Directive which specifies that killing must beperformed by an "adequately educated and trained person" using an approved method (European Parliament and Council of the European Union 2010). Althoughcephalopods are included in the Directive, no specific recommendations of methodsfor killing are listed in Annexe IV. In view of this we have applied the general principles outlined in Annexe IV (1a) to cephalopods (Andrews et al., 2013; Fiorito et al., 2015)that, when killed the animal should be unconscious, should remain unconscious untildeath ensues and is confirmed. In all experiments included here the animals fulfilled

the criteria for general anaesthesia (as mentioned above, and see: Andrews and Tansey 1981; Andrews et al.,2013; Gleadall 2013; Fiorito et al., 2015) when killed.

4.3 Killing methods

Depending upon the protocol, the animals were exposed to one of the following treatments:

- **$MgCl_2$:** immersion for 20min in 2L of 3.5% magnesium chloride dissolved in sea water (sw) at room temperature (18-21°C). At this time the animals are immobile, pale, lack a righting reflex, are unresponsive to handling and ventilation is suppressed or absent (see review of criteria in Fiorito et al., 2015).
- **$MgCl_2$ (1:1):** immersion for 20min in 2L of 3.5% magnesium chloride dissolved in a mixture of sea-and distilled-water (1:1, sw:dw as in Pagano et al., 2011). After 20min the appearance of the animal was similar to the above description and to that in Messenger et al. (1985). Not that similar to Pantin (1946), Messenger et al., (1985) mixed 7.5% $MgCl_2$ dissolved in distilled water with an equal volume of sw to achieve a final concentration of 3.75 % $MgCl_2$
- **Mix:** immersion in a 2L mixture of magnesium chloride (1.12%) and ethanol (1%) in sea water. Exposure was either for 20min (Mix 20') or 45min (Mix 45') to match the times used by some authors for achieving anaesthesia in *O. vulgaris* for neurophysiological studies (i.e. 55mM $MgCl_2$and 1% ethanol in sw: Shomrat et al., 2008) or other purposes (Pagano et al., 2011). Exposure times of 25-45min to a mixture of $MgCl_2$(1.12%) and ethanol (1%) have also been used for induction of deep anaesthesia in *O. vulgaris* (Shomrat et al., 2008; 2011).
- **Hypothermia:** immersion in 2L of sea water at 4o 215 C for 5-10 minutes. Profound cooling of Mediterranean *O. vulgaris* has been used to 'anaesthetize' animals when the use of a chemical agent could compromise the experimental outcome (Andrews et al., 1981). Although there is debate (Gleadall 2013) about the exact effects of cooling these animals well outside their normal thermal range (see also discussion in Agnisola et al., 1996), cooling has been reported to produce a state comparable to the one induced by the anaesthetic agent urethane and ethanol (Andrews and Tansey, 1981).

After treatments, the brain was destroyed to complete killing as described in Directive 2010/63/EU Annexe IV (2b) (see also Andrews et al., 2013; Fiorito et al., 2015).

4.4 Osmolality measurements

The osmolality of the sea water, perfusion medium and the anaesthetic formulations used for the experiments was measured using an autocal Osmometer (Roebling) according to recommendations for the use of osmometry methods for biological samples (Sweeney and Beuchat 1993). For each solution four samples were measured 322 (at 20°C) in triplicate.

4.5 Chemicals and Solutions

4.5.1 Physiological studies

Glucose (CAS Number: 50-99-7) and magnesium chloride hexahydrate (CAS Number: 7791-18-6) was purchased from SIGMA ALDRICH; Ethanol (99%, CAS Number 64-17-5) was purchased from APPLICHEM.

4.5.2 Molecular studies

Protease Inhibitor Cocktail (p8340), Tris-Base (CAS Number 77-86-1), $NaCl_2$ (CAS Number 7647-14-5), IGEPAL CA-630 (CAS Number 9002-93-1), Sodium deoxycholate (CAS Number 302-95-4), EDTA (CAS Number 60-00-4) were purchased from SIGMA ALDRICH; Bovine Serum Albumin (BSA) was purchased from Cell Signaling; Acrylamide, Prestained protein molecular weight markers and other electrophoretic materials were purchased from Bio-Rad (Richmond, CA).

The buffers used had the following composition:

Lysis buffer: Tris-Base (50 mM), NaCl2 (150 mM), IGEPAL (1%), Sodium deoxycholate (0.5%), EDTA (1 mM), SDS (0.1%), pH 7.5 and added of a mixture of protease inhibitors containing AEBSF at 104 mM, Aprotinin at 80 µM, Bestatin at 4 mM, E-64 at 1.4 mM, Leupeptin at 2 mM and Pepstatin A at 1.5 mM).

Running buffer: Tris-Base (25mM), Glycine (250 mM), SDS (0.1%), pH 8.3;

Western buffer: Tris-Base (48mM), Glycine (39mM), SDS (0.1%), methanol (20%), pH 8.3;

Washing solution: Tris Buffered Saline, TBS, NaCl2 (137 mM), Tris-HCl (15mM), pH 7.6) added with Tween-20 0.1% (TBST).

Polyclonal antibodies: anti-HSP70 (K-20: sc-1060), anti-Actin (H-300, sc-10731) (Santa Cruz Biotechnology, INC), anti-p38 MAPK #9212, anti-SAPK/JNK #9252, anti-phospho-SAPK/JNK (Thr183/Tyr185) #9251 (Cell Signaling).

Monoclonal antibodies: anti-Caspase-3 (8G10) #9665, anti-phospho-p38 MAPK (Thr180/Tyr182) #4511 (Cell Signaling).

Goat anti-rabbit IgG-HRP conjugated (sc-2004), bovine anti-goat IgG-HRP conjugated (sc-2350) were from Santa Cruz Biotechnology, INC.

4.6 Systemic heart isolation

Immediately after brain destruction, the systemic heart was isolated according to Foti et al. (1985) and Houlihan et al. (1987). The heart dissection was performed at 4°C and took ~ 15 minutes.

In *O. vulgaris*, blood enters the heart through two auricles and leaves the ventricle through three arteries: the dorsal (cephalic) aorta and the abdominal and gonadial arteries. A rich network of coronary veins is present above the surface of the heart which drain the blood directly from the ventricular lumen during ventricular systole (Agnisola et al., 1990). Both the auricles and the dorsal aorta were cannulated while the gonadial and abdominal aortae were ligatured at their base. The outflow from the coronary veins passed out into the chamber and was collected by an overflow system. The fusiform ganglion was removed.

The heart was connected to the perfusion apparatus where the two auricles received perfusion fluid at the same controlled input pressure (**Fig. 3**).

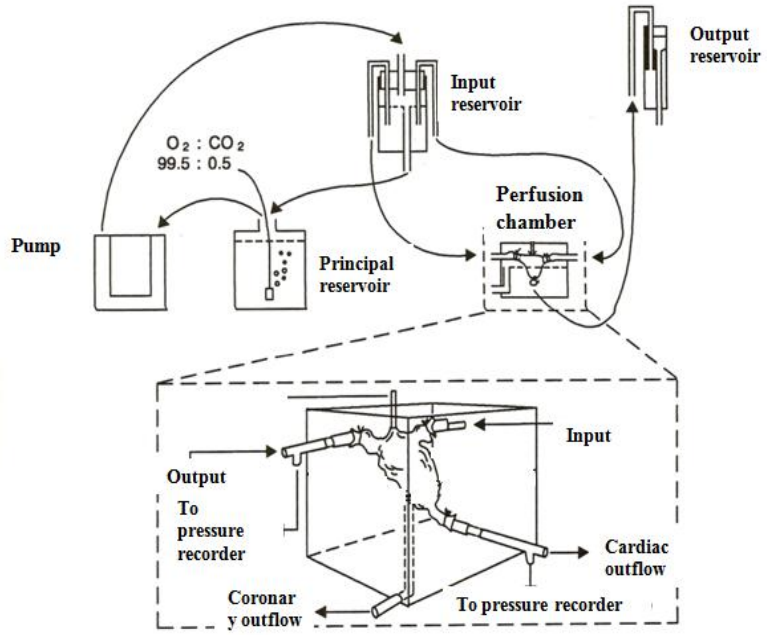

Fig. 3. Perfusion apparatus for *Octopus vulgaris* heart (from Venzi R., 1992)

The perfusion solution (PS) contained filtered sea water containing 2.78 mM anhydrous glucose, gassed with 99.5% oxygen and 0.5% carbon dioxide. The pH of the perfusion medium was 8.0 (Houlian et al., 1987; Agnisola et al., 1989, Agnisola, 1994) and is close to the pH of *O. vulgaris* haemolymph (medium value 7.8±0.1: D'Aniello et al., 1986).

The basal perfusion conditions were chosen to reproduce *in vivo* resting hemodynamic parameters as previously reported (Agnisola et al. 1989; 1994). The preload (input pressure) was adjusted to obtain a stroke volume (SV) of 0.7-0.8 ml/g of ventricular weight, a value that is approximately physiological for resting animals of a similar size to those used here (Wells and Smith, 1987). The diastolic output pressure was always set at 2kPa above the preload which corresponds to the diastolic aortic pressure in resting animals (Wells and Smith, 1987). Experiments were performed at room temperature (18-21°C). The heart beats spontaneously and does not require pacing at this temperature. Under these conditions, aortic pressure, heart rate, aortic and coronary flow were measured for 15-20 min to assess their stability and if parameters had not stabilised within the range established in previous studies the experiment was not continued. About 15% of hearts failed to meet the criteria for stabilisation but there was no obvious relationship between body weight, sex and anaesthetic protocol and failure to stabilize.

4.7 Measurements and calculations

Preload and afterload (kPa) were defined as the mean input and output pressures, respectively. The pressure measurements were referred to the level of perfusate in the perfusion chamber and corrected for cannulae resistance. Pressures and heart rate (HR, beats/min) were measured through two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA) connected to a PowerLab data acquisition system and analyzed using Chart software (AD-Instruments, Ugo Basile, Comerio, Italy). Other cardiac parameters are measured as follows:

- **Cardiac output**(*Q,* ml/min/g) and Coronary output (CorO, ml/min/g) were derived from the dorsal aorta outflow and coronary vein outflow, respectively, collected over 1 min and weighed. Values were corrected for fluid density and normalized per gram ventricle wet weight.
- **Stroke volume** (SV, ml/g) was calculated from *Q*/HR and used as an index of contractility.
- **Stroke work** (SW, mJ/g) was calculated as (afterload-preload) x SV/ventricular wet weight.
- **Power output** (PO, mW/g) was calculated as: (afterload-preload) x *Q*/60

The separation between aortic and coronary output is necessary because during contraction, a proportion of the total cardiac output is collected in the coronary veins passing through the walls of the heart (Foti et al., 1985; Houlihan et al., 1987; Agnisola et al., 1994). Thus, contraction of the isolated ventricle resulted in ejecting flow through the cannulated aorta (i.e. aortic output) and through the walls of the heart, into the cut coronary veins (i.e. coronary output). In figures *Q*, SV, and SW are related to aortic output.

4.8 Statistics

Physiological results are expressed as mean ± SEM of percentage changes obtained from individual experiments unless otherwise stated. Statistical analysis was performed on raw data (not %) following Zar (1999). All data were tested for normality. We utilized repeated-measures ANOVA followed by Bonferroni's Multiple Comparison test whenever appropriate. Multivariate Analysis of Variance (MANOVA) was utilized to test effects of different treatments for Frank-Starling curves. Mixed Model ANOVA was utilized as method for testing effects of repeated-measures when the sample numbers were not matched for all treatments/parameters. Differences were considered statistically significant at $p < 0.05$. For all statistical analyses we used SPSS (rel. 14.0, SPSS Inc - Chicago, 2005).

For densitometric analyses, values were expressed as means ± SEM of absolute values from individual experiments; statistic was assessed by One-way ANOVA followed by Bonferroni multiple comparison test ($p < 0.05$). GraphPad Software, version 5, San Diego, CA was used for the statistical analysis.

5. Does *in vivo* exposure to $MgCl_2$ formulations have residual effects on the heart *in vitro*?

5.1 Experimental procedure

Following isolation of the systemic heart from animals (n = 27), immersed in the different magnesium formulations [n = 5 for $MgCl_2$; n = 5 for $MgCl_2$(1:1); n = 5 for Mix 20'; n = 6 for Mix 45'] or hypothermia [n = 6 for hypothermia (4°C)], Frank–Starling curves were generated to assess the effect of these treatments on the cardiac sensitivity to increased preloads. The Frank-Starling mechanism (heterometric cardiac regulation) is a property of the myocardium to respond to increased venous return (preload) with a more forceful contraction (contractility) of its lengthened fibres, thus increasing SV and hence *Q*.

After an initial period of stabilization (15-20 min) at baseline conditions, the input pressure was increased stepwise in 0.1 kPa increments until the maximal cardiac output was reached. Each pressure increment was maintained for 5 min during which cardiac parameters were evaluated.

In addition to physiological studies, in order to determine if anaesthetic treatments used could represent a stress factor, biological studies were performed. Protein sample was pooled from three systemic hearts of *O. vulgaris* (obtained from animals after exposure to the different anaesthetic treatments) to evaluate the expression of HSP70 and caspase, and the phosphorylation of JNK and p38 MAPKs.

5.2. Results

5.2.1 Osmolality measurements

Osmolality values of the solutions used for *in vivo* (by immersion) studies are reported in **Table 3**.

The osmolality of sea water was 1125.25±1.39mOsm/Kg (n=4) and the osmolality of the perfusion solution (sea water+glucose) was 1139.67±1.17 mOsm/Kg (n=4).

Table 3. Osmolality of the anaesthetic solutions utilized to anaesthetize the animals. Values are derived from measurements from three replicates of four samples for each solution. For each formulation the first row refers to the concentration (%, in italics) of substances utilized and the second to the mean (± SEM) osmolality calculated for each solution.

Solutions	%	Osmolality (mOsm/Kg)
$MgCl_2$	**3.5**	1605.92±3.31
$MgCl_2$ (1:1)	**3.5**	1009.92±3.93
Mix (20' 45')	**1.12/1**	1481.42±2.72

1. for all cases, % of $MgCl_2$ precedes the % value of EtOH utilized

5.2.2 Baseline haemodynamic parameters in the isolated heart

The baseline haemodynamic parameters in the spontaneously beating isolated hearts from animals with the five different pre-isolation treatments are summarised in **Table 4** Irrespective of the different anaesthetic treatments (including hypothermia), at baseline conditions the perfused hearts were able to produce physiological values of stroke volume and to work at hemodynamic loads comparable with those reported by other authors for *in vitro* preparations (Agnisola et al., 1989; Agnisola and Houlihan 1994). No differences were detected in baseline parameters of hearts with different treatments, with exception of afterload pressure and CorO (**Table 4**). Although the afterload values were in the physiological range (see as reference: Wells et al., 1987; Wells and Smith 1987) differences emerged only when comparing $MgCl_2$ vs hypothermia and $MgCl_2$ vs Mix 45' (Table 3). In addition, the coronary outputs were only significantly different when comparing hypothermia vs $MgCl_2$ (1:1) or Mix 20' (**Table 4**).

Table 4. Baseline hemodynamic parameters of *O. vulgaris* isolated hearts after stabilization. Values are plotted as mean ± SEM (N=number of animals per treatment). HR: heart rate; Q: cardiac output; CorO: coronary output; SV: stroke volume; SW: stroke work; PO: power output. One-way ANOVA (Afterload): F(4,22) = 5.07, p = 0.005, after Bonferroni's Multiple Comparison Test (*):$MgCl_2$ vs hypothermia, p = 0.031; $MgCl_2$ vs Mix 45', p = 0.006. One-way ANOVA (CorO): F(4,22) = 4.17, p = 0.012, after Bonferroni's Multiple Comparison Test (*), hypothermia vs $MgCl_2$ (1:1), p = 0.031; hypothermia vs Mix 45', p = 0.012; hypothermia vs Mix 20', p = 0.054 (marginally significant).

Anaesthetic treatment	Preload (kPa)	Afterload (kPa)	HR (beats/min)	*Q* (ml/min/g)	CorO (ml/min/g)	SV (ml/g)	SW (mJ/g)	PO (mW/g)
Hypothermia (n=6)	0.33 ± 0.03	24.13 ± 0.55$^{*,\$}$	23.50 ± 1.85	17.99 ± 1.45	3.13 ± 0.78	0.77 ± 0.03	1.79 ± 0.07	0.69±0.04
$MgCl_2$ (n=5)	0.4 ± 0.06	29.2 ± 0.96 *	29.2 ± 3.3	19.42 ± 1.17	4.58 ± 0.46	0.68 ± 0.05	1.94 ± 0.18	0.91±0.06
$MgCl_2$ (1:1) (n=5)	0.32 ± 0.03	27.1 ± 1.07	22.8 ± 2.2	21.79 ± 2.71	4.97 ± 0.32	0.75 ± 0.07	1.97 ± 0.19	0.95±0.11
Mix 20' (n=5)	0.37 ± 0.05	24.9 ± 1.75	29.8 ± 3.15	19.45 ± 1.33	4.77 ± 0.45	0.68 ± 0.06	1.63 ± 0.11	0.77±0.05
Mix 45' (n=6)	0.49 ± 0.04	23.08 ± 0.98$^{\$}$	29.0 ± 4.00	19.38 ± 3.70	5.54 ±0.68	0.68 ± 0.08	1.51 ± 0.20	0.70±0.11

5.2.3 Frank–Starling curves

Frank-Starling curves obtained from cardiac preparations of animals treated with $MgCl_2$, $MgCl_2$ (1:1), Mix 20', Mix 45', and hypothermia showed that, with increasing preload, a significant raise in Q, SV and SW occurred (**Fig. 4**).

The preload increases did not produce a significant change in the heart rate in any of the treatments, thus the increases in cardiac output were due solely to an increase in stroke volume. The Frank-Starling curves for the individual treatments are plotted in **Fig. 4**. Hearts isolated from animals under either hypothermia or $MgCl_2$ in sea water endured a higher number of pre-load increments (6 increments) before reaching the plateau of the Frank-Starling curve (i.e. the optimal preload at which point Q remains constant) followed by Mix (20') and $MgCl_2$ (1:1) (5 increments), and Mix 45' (4 increments).

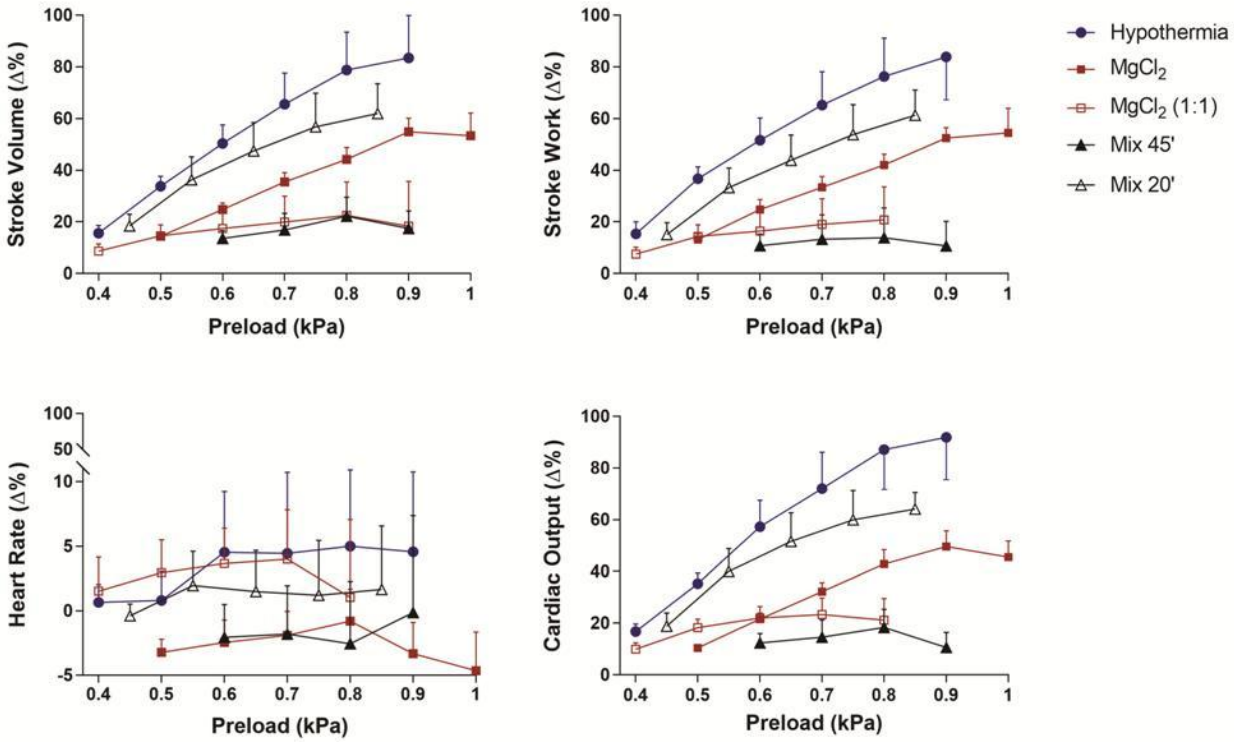

Fig. 4. Effect of preload increase on stroke volume (SV), stroke work (SW), heart rate (HR) and cardiac output (Q) in the isolated *Octopus vulgaris* systemic heart (n = 6 each treatment) following removal under hypothermia (4°C), $MgCl_2$, $MgCl_2$ (1:1), and $MgCl_2$+EtOH mixture following 20 (Mix 20') and 45 (Mix 45') minutes of exposure.
Results are expressed as mean ± SEM of the percentage (%) change from baseline (see Table 4). Repeated-measures ANOVA revealed no significant differences among treatments, but treatments x preload effects were significant with the exception of HR (SV: $F(4,16) = 2.16$, $p = 0.121$; treatments x preload $F(24,96) = 3.29$, $p < 0.001$; SW: $F(4,16)$ 2.62, $p = 0.074$; treatments x preload $F(24,96) = 4.42$, $p < 0.001$; HR: $F(4,16) = 0.84$, $p = 0.518$; treatments x preload $F(24,96) = 0.31$, $p = 1.000$; Q: $F(4,16) = 0.36$, $p = 0.831$; treatments x preload $F(24,96) = 6.14$, $p < 0.001$. MANOVA was utilized to evaluate pair wise differences in preload values between $MgCl_2$ (1:1) and (Mix 45') and the other curves (see text for details).
The largest response in terms of Q, SV, and SW was obtained with hypothermia followed by treatment with Mix 20' and $MgCl_2$. Hypothermia, Q = 91.8 ± 17.2%; SV = 83.4 ± 14.9%; SW = 83.9 ± 15.9%; $MgCl_2$, Q = 49.6 ± 4.3%; SV, 54.9 ± 3.7%; SW = 52.5 ±2.9%; Mix 20', Q = 64.1 ± 7.4%; SV = 61.9 ± 11.5%; SW = 56.5 ± 10.5%. $MgCl_2$ (1:1) and Mix 45' showed the worst response revealed by an impaired ability to respond to preload increases [$MgCl_2$ (1:1): Q = 23.3 ± 6.4%; SV = 22.5 ± 11.5%; SW = 20.8 ± 11.4%; Mix 45', Q = 22.9 ± 4.0%; SV = 22.2 ± 7.4%; SW = 13.9 ± 11.5%].

The individual Frank-Starling curves (**Fig. 4**) for each pre-isolation treatment revealed that over a similar pressure range the curves for $MgCl_2$ (1:1) and Mix 45' were flatter than those for all other pre-isolation treatments. This is confirmed by the maximum values reached for all cardiac functions parameters (see legend in **Fig. 4**). At the maximal input pressure used (until there was no further increase in Q), the largest response in terms of Q, SV, and SW was obtained with hypothermia followed by treatment with Mix 20' and $MgCl_2$. $MgCl_2$ (1:1), and Mix 45' showed the worst response revealed by an impaired ability to respond to preload increases (**Fig. 4**). Repeated-measures ANOVA confirmed this view, revealing no significant differences between any of curves for each treatment for any of the parameters considered (SV, SW, HR, and Q, see Fig. 3), but also highlight a significant treatment*preload effect for all parameters considered ($p < 0.001$), with the exception of HR ($p = 0.999$), thus confirming that the hearts all responded to increases in preload. Inspection of the Frank-Starling curves (**Fig. 4**) revealed that hearts isolated under hypothermia, Mix 20' and $MgCl_2$ were all capable of increasing stroke volume by ~50% or more, whereas this was not the case for the $MgCl_2$ (1:1) and Mix 45' hearts. Bonferroni's Multiple Comparison Tests after Multivariate Analysis of Variance (MANOVA) confirmed this view. Significant differences in the preload-stroke volume relationship were observed only for hypothermia vs Mix 45' (preload values 0.6, 0.8, 0.9 kPa, $p < 0.05$) and for hypothermia vs $MgCl_2$ (1:1) only at the two highest preloads ($p < 0.05$). A similar view emerged considering stroke work where significant differences resulted for hypothermia vs Mix 45' ($p < 0.05$) and $MgCl_2$ vs Mix 45' ($p < 0.05$, only at the last preload value). No other significant differences emerged when considering other parameters. Figure 4 also shows that the Frank-Starling curves for hypothermia, Mix 20' and $MgCl_2$ are parallel to each other; in addition, the responses of the hearts isolated under $MgCl_2$ (1:1) and Mix 45' are similar to each other. In the latter the preload-stroke volume relationship was shallow in comparison to that in hearts isolated under hypothermia, Mix 20' or $MgCl_2$. These differences in slope are also reflected in calculations of the change in stroke volume per kPa change in input pressure (δ kPa) using the values from the individual Frank-Starling curves; for hypothermia (n=6): 2.01 ± 0.21 ml/g/kPa; for $MgCl_2$ (n=5): 1.4 ± 0.10 ml/g/kPa; for $MgCl_2$ (1:1; n=5): 1.01 ± 0.16 ml/g/kPa; for Mix 20' (n=5): 1.52 ± 0.14 ml/g/kPa; for Mix 45' (n=6): 1.08 ± 0.18 ml/g/kPa.

6. Does *in vivo* exposure to $MgCl_2$ formulations induce stress response in cardiac tissue?

A cellular mechanism for coping with stress is the expression of a group of proteins designated as heat shock proteins (HSPs). These proteins represent a family of highly conserved protein found in different organisms from bacteria to humans, which act as molecular chaperones (Ellis and van der Vies, 1991) ensuring stabilization and proper folding of newly synthesized proteins and preventing protein aggregation of denatured proteins (Fink, 1999). More precisely, the HSP70 and HSP60 families intervene in the folding, assembling and translocation of proteins among intracellular compartments (Morimoto et al., 1990). Furthermore, in addition to its chaperone functions in protein folding and assembly, HSP70 protects cells from a number of apoptotic stimuli.

HSP70 family appears to be the most evolutionary preserved and distributed in animals (De Maio, 1999; Feder and Hofmann, 1999), expressed in unstressed cells as constitutive proteins (HSC70) or as stress-inducible forms (HSP70) (Hartl and Hayer-Hartl, 2002). Also in molluscs, many types of stress, such as exposure to heat and cold, organic pollutants, heavy metals, oxidants, UV light, hypoxia, salinity and other physical or chemical stressors induce HSPs expression (Sanders, 1993; Clegg et al., 1998; Piano et al., 2004; Anestis et al., 2007, 2008; Gonzalez-Riopedre et al., 2007; Ivanina et al., 2009; Snyder et al 2001).

While HSP70 is an antiapoptotic protein, the caspase-mediated apoptotic death induced by diverse stressful conditions is well established in a plethora of mammalian cell types (for a review, see Bredesen et al., 2004; Jiang and Wang, 2004; Philchenkov, 2004).

However, very little is known on the mechanisms that lead to apoptotic cell death in marine invertebrates. Also the intracellular signal transduction pathways involved in the response of cephalopods to different environmental challenges are still unknown.

In most eukaryotic cells, from yeasts to mammals, various forms of cellular stress lead to the activation of conserved intracellular enzymes, i.e. the mitogen activated protein kinases (MAPKs) (Schaeffer and Weber, 1999; Widmann et al., 1999; Kyriakis and Avruch, 2001). Three subfamilies of the MAPKs have been clearly identified: the extracellularly responsive kinases (ERKs), the c-Jun NH2-terminal kinases (JNKs), and the p38-MAPKs.

The ERK pathway is mainly activated by mitogen or differentiation inducing agents, whereas JNKs and p38 MAPK pathways are typically responsive to several stresses

(Kyriakis and Avruch, 2001). In particular, p38-MAPK has been characterized as the principal stress-kinase responsive to fluctuations in ambient osmolality and temperature (mammals: Zhang and Cohen, 1996; Gon et al., 1998; Sheikh-Hamad et al., 1998; marine invertebrates: Gaitanaki et al., 2004; Kefaloyianni et al., 2005; Anestis et al., 2007; Gourgou et al., 2010).

Evidence from mammalian cell systems also indicates that the activity of p38 MAPK and cJun-N-terminal kinases (JNKs) is essential for HSP expression during various cell stresses (Rafiee et al., 2003; Sheikh-Hamad et al., 1998; Uehara et al., 1999). Recently, studies on *M. galloprovincialis* revealed the involvement of the p38-MAPK in the pro- or anti-apoptotic event in response to thermal and heavy metal stress; identical stressful stimuli possibly lead to apoptotic death via the caspase-3 activation in the mantle tissue and to anti-apoptotic events possibly *via* the induction of HSP70 overexpression (Kefaloyianni, et al., 2005). In addition, MAPKs (p38 and JNK) signaling might be involved in the regulation of HSP expression in *M. galloprovincialis* to increased temperature (Anestis et al., 2007; Gourgou et al., 2010).

To our knowledge, there is no evidence relating MAPKs activation and HSP70 expression in the tissues of cephalopods after exposure to anesthetic treatments. Considering that the MAPKs family may play an important role in coordinating protein expression to various stresses, we examined the phosphorylation and hence activation of stress-activated protein kinases, p38 MAPK and JNKs, in the systemic hearts of *O. vulgaris* after exposure to anesthetic treatments.

6.1 Experimental procedure

Samples were homogenized in ice-cold lysis buffer, using an ultra-turrax homogenizer and aliquots were centrifuged at 10000g at 4°C for 10 min. Protein concentration was determined by using Bradford reagent, according to the manufacturer (Bio-Rad). SDS-PAGE and Western blotting analysis were performed according to the Laemmli (1970) procedure. Equal amounts of proteins were separated on 10% SDS-PAGE gels (for JNK, p-JNK, p38 MAPK and p-p38 MAPK detection, 60 μg) or on 12% SDS-PAGE gels (for caspase-3, 60 μg). A mixture of proteins with known molecular weights was used as molecular weight markers.

After electrophoresis, proteins were trans-blotted onto nitrocellulose membrane using a Trans-blot chamber (Bio-Rad) for 2 hr at 60 V. The blots were incubated in Tris-buffer saline (TBS), additionated with the Tween 20 (TBS-T), containing 5% non-fat dry milk or BSA to block non-specific binding sites.

Incubation with primary antibody at a 1:1000 dilution was performed overnight at 4°C. After washing with TBS-T, the blots were incubated with the peroxidase linked secondary antibody at 1:2000 dilution for 1 h at room temperature. Protein loading was verified by using total p38 MAPK for p-p38 MAPK, and total JNK for p-JNK; Actin was used as control screen to normalize HSP70 loading. Protein bands were visualized by using an enhanced chemiluminescence kit (ECL, GE Healthcare, Amersham). Autoradiographs were obtained by exposure to X-ray films (Hyperfilm ECL, Amersham). Densitometric analysis of the bands was carried out using the ImageJ software.

6.2 Results

6.2.1 Evaluation of stress response after in vivo exposure to magnesium chloride formulations

Expression of HSP70 and caspase-3, and the phosphorylation of JNK and p38 MAPK were determined in cardiac homogenates, obtained from samples of *O. vulgaris* immersed in different magnesium formulations or cold sea water (4°C).

- HSP70 expression was revealed in all samples used. In particular, its expression was higher in the hearts from animals treated with magnesium formulations compared to hypothermia. Moreover, among different magnesium formulations, Mix 45' showed significant differences respect to other treatments in the HSP70 expression probably due to the prolonged exposure time (**Fig. 5**).

- The expression of HSP70 may play a cytoprotective role in the stress response during anaesthesia. This latter finding is also supported by the lack of caspase-3 expression in all analyzed tissues which excludes pro-apoptotic events by all anaesthetic treatments (data not shown).

- As no significant differences in the HSP70 expression were revealed among the different treatments ($MgCl_2$, 1:1, Mix), a role of different osmolality was in the observed effects, may be excluded (**Fig. 5**).

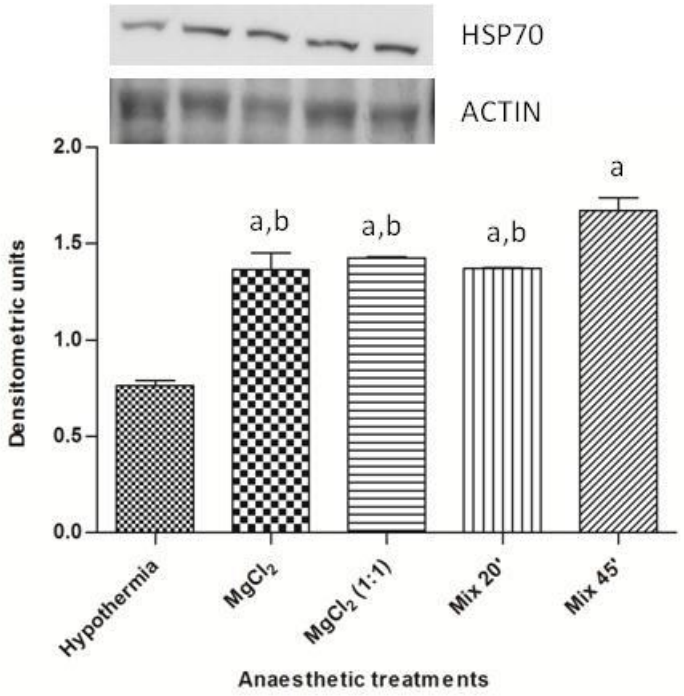

Fig. 5. Western blotting and densitometric analysis of HSP70 expression in systemic hearts of *O. vulgaris* under different anaesthetic conditions. Equal protein loading was confirmed by blotting identical samples with an anti-actin specific antibody. Western blot shown is representative of 3 independent experiments. One-way ANOVA followed by Bonferroni multiple comparison test were performed for group comparisons. $^{a}p < 0.05$ vs. hypothermia, $^{b}p < 0.05$ vs. Mix 45'

- Animals exposure to the different anaesthetic treatments induced a phosphorylation pattern of cardiac p38 and JNK MAPKs (**Fig. 6-7**). The activity of the two MAPKs significantly increased under all magnesium chloride formulations compared to hypothermia. The increased phosphorylation of p38 and JNK MAPKs paralleled with the increased expression of HSP70 may support the involvement of MAPK signaling cascade in the expression of HSP70.

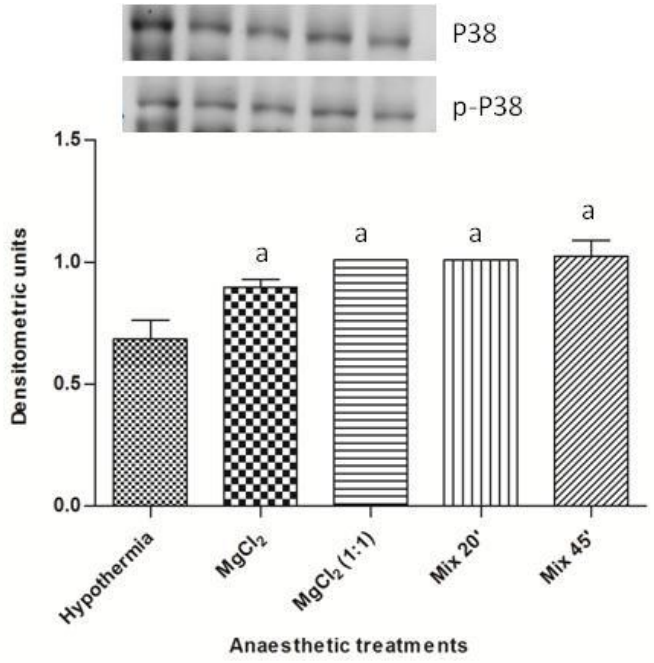

Fig. 6. Western blotting and densitometric analysis of phosphorylation levels of phospho p38 (p-p38/p38) in systemic hearts of *O. vulgaris* under different anaesthetic conditions. Western blot shown is representative of 3 independent experiments. One-way ANOVA followed by Bonferroni multiple comparison test were performed for group comparisons. [a]$p < 0.05$ vs. hypothermia.

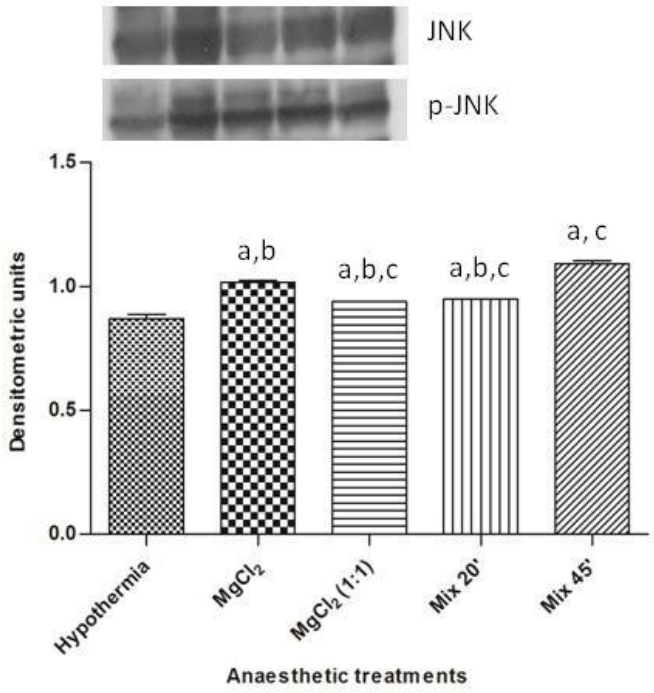

Fig. 7. Western blotting and densitometric analysis of phosphorylation levels of phospho JNK (p-JNK/JNK) in systemic hearts of *O. vulgaris* under different anaesthetic conditions. Western blot shown is representative of 3 independent experiments. One-way ANOVA followed by Bonferroni multiple comparison test were performed for group comparisons. [a]$p < 0.05$ vs. hypothermia; [b]$p < 0.05$ vs. Mix 45'; [c]$p < 0.05$ vs. $MgCl_2$.

7. What direct effects do $MgCl_2$ formulations have on cardiac function?

7.1 Experimental procedure

The systemic heart was isolated from animals (n = 23) killed following hypothermia (see above). After stabilization of cardiac parameters (15-20 min) during which the hearts were perfused with PS (see above), cumulative concentration-response curves were generated to evaluate the action of the different anaesthetic regimes on cardiac function with the agents delivered in PS. The formulations tested were: $MgCl_2$ (n = 7) and $MgCl_2$ (1:1, n = 5) at 0.25%, 0.5%, 1%, 2%, and Mix (n = 6) at 0.14/0.125 %, 0.28/0.25 %, 0.56/0.5 %, 1.12/1%. Ethanol alone (n = 5) at increased concentrations (0.125 %, 0.25 %, 0.5 %, 1 %) was also tested to separate the effects of $MgCl_2$ from ethanol in the mixture. Each concentration was tested for 10-15 minutes.

7.2 Results

7.2.1 Osmolality measurements

Osmolality values of the solutions used *in vitro* (by perfusion) are reported in **Table 5**.

Table 5. Osmolality of the anaesthetic solutions utilized for *in vitro* heart perfusion. Values are derived from measurements from three replicates of four samples for each solution. For each formulation the first row refers to the concentration (%, in italics) of substances utilized and the second to the mean (± SEM) osmolality calculated for each solution.

Solutions	**%**	**Osmolality (mOsm/Kg)**			
	%	**0.25**	**0.5**	**1**	**2**
$MgCl_2$		1152.67±2.79	1186.17±1.7	1254±2.47	1410.67±3.14
	%	**0.25**	**0.5**	**1**	**2**
$MgCl_2$ (1:1)		585.33±1.10	617.83±1.80	694±3.83	831.58±1.94
	%	**0.14/0.125**	**0.28/0.25**	**0.56/0.5**	**1.12/1**
Mix		1163.67±1.39	1212.67±0.94	1310±2.56	1481.42±2.72
	%	**0.125**	**0.25**	**0.5**	**1**
Ethanol		1144.17±1.51	1169.5±1.53	1215.58±2.85	1313.33±1.67

1. for all cases, % of $MgCl_2$ precedes the % value of EtOH utilized

7.2.2 Anaesthetic concentration-response curves

Perfusion with increasing concentrations of different anaesthetic solutions was carried out on systemic hearts removed under hypothermia and the effects on HR, SV, and Q are plotted in **Fig. 8**.

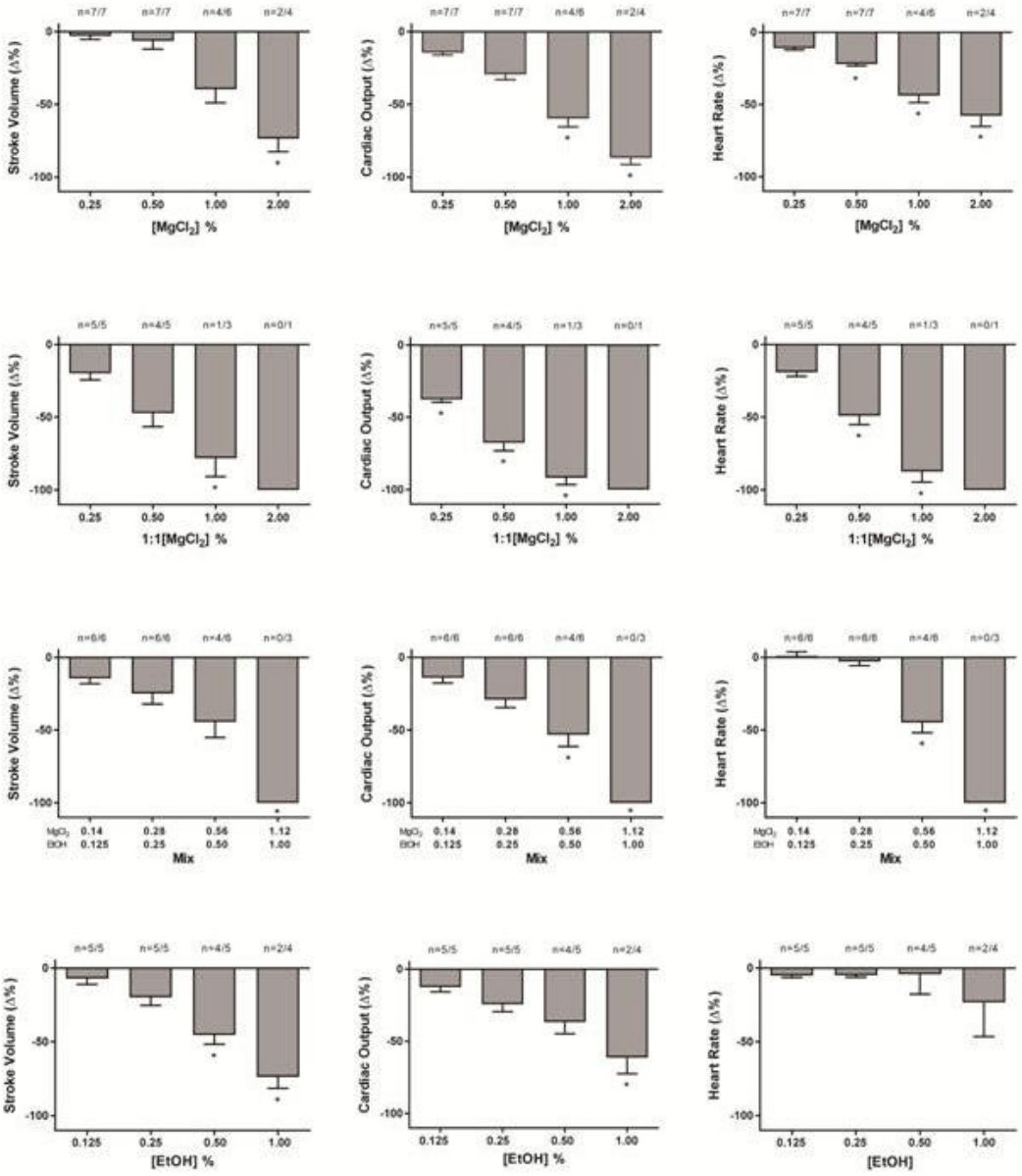

Fig. 8. Effect of increasing concentrations of $MgCl_2$, $MgCl_2$ (1:1), Mix, and ethanol on the percentage change from baseline values in heart rate (HR), stroke volume (SV) and cardiac output (Q); values are presented as mean ± SEM percentage (%) change. The number of hearts (n, beating/tested) are indicated above each column. Significant differences are marked by (*, $p < 0.05$). Note that at the same concentration of $MgCl_2$, the effects of the 1:1 formulation are greater than the sea water formulation. In addition, whilst both Mix and ethanol affect the stroke volume and cardiac output, the effects of ethanol alone on heart rate are less marked (0.5% and 1%) in comparison to the same

concentration of ethanol mixed with magnesium chloride. See text for details.

All anaesthetics, except ethanol, induced bradycardia in a concentration-related manner. In particular, $MgCl_2$ and Mix significantly reduced HR, Q, and SV ($p < 0.01$ after Mixed Model ANOVA, except HR with EtOH) at the higher concentrations tested (**Fig. 8**). $MgCl_2$ (1:1) appears to be a more potent cardio-suppressive agent than either $MgCl_2$ or Mix because it induced a significant reduction of cardiac parameters starting at a lower $MgCl_2$ concentration. All anaesthetic solutions were able to produce cardiac arrest at the higher concentrations used (**Fig. 9**) with the incidence of arrest lowest with $MgCl_2$ and ethanol alone. In all cases the heart could be restarted in <10 min, when re-perfused with PS alone (data not shown).

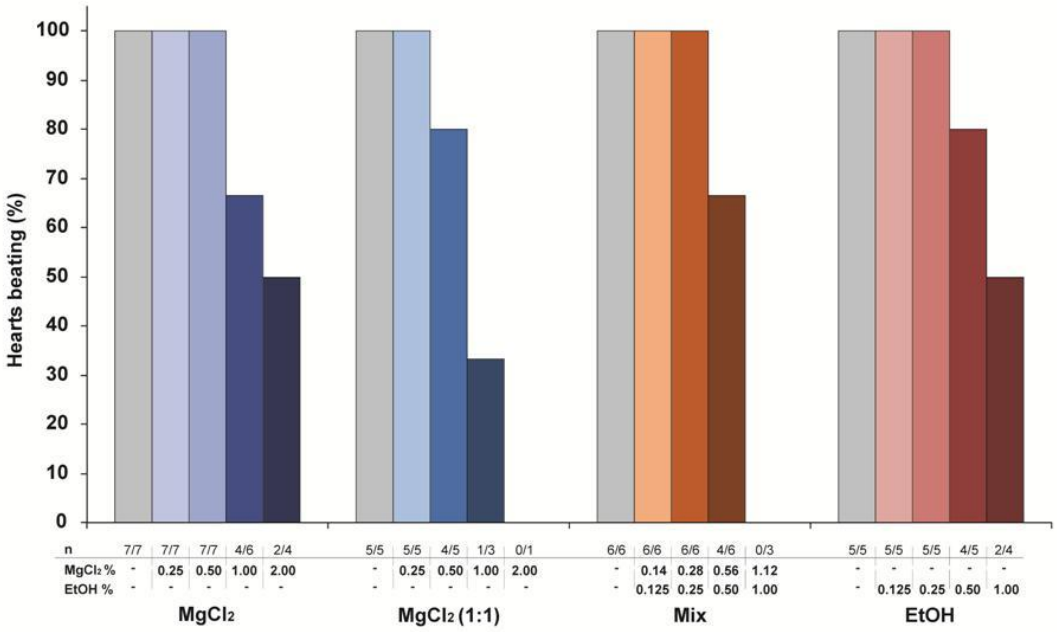

Fig. 9. The effect of cardiac perfusion with increasing concentrations of anaesthetic solutions on the incidence of spontaneous beating in hearts isolated under hypothermia (see Methods for details). The number of hearts treated is lower at the higher concentrations, as fewer studies were conducted at concentrations above the threshold where arrest was first observed.

8. Discussion

8.1 Basal conditions

The *in vitro* working preparation of the systemic heart is able to generate physiological values of cardiac output, output pressure, ventricle work and power in various cephalopod preparations, e.g.: *S. officinalis* (Kling and Jakobs 1987; Jakobs and Schipp 1992; also perfused *in situ* in MacCormack et al., 2016), *E. cirrhosa* (Smith 1981), and *O. vulgaris* (Foti et al., 1985; Houlihan et al., 1987; Agnisola et al., 1989; Agnisola and Houlihan, 1994).

Baseline values of cardiac function from both hypothermic and magnesium-treated animals (**Table 4**) are similar to those reported for isolated hearts perfused with oxygenated saline (Agnisola and Houlihan, 1991). Perfusion conditions replicated physiological resting afterload and stroke volume values as previously reported (Agnisola et al., 1989, 1994). All preparations were stable for 70-100 minutes, a time comparable to Agnisola et al. (1994). Other authors reported a shorter period of viability (about 50 min, Houlihan et al., 1987), followed by a notable decline in cardiac output, probably due to the higher input pressures used (0.5-2.0 kPa, Houlihan et al., 1987). *In vitro* baseline input pressures were higher than those reported *in vivo* (0.05-0.25 kPa in the efferent branchial vessel, Wells and Smith 1987), but similar to those previously reported *in vitro* in *O. vulgaris* (0.49 kPa, Smith 1981; Houlihan et al., 1987; 0.23 kPa, Agnisola et al., 1989). The mean afterload was within the physiological range: resting values of 2.0 kPa in diastole or 3.5 kPa in systole are typical of animals in the 400- 1000g body weight range (Wells et al., 1987; Wells and Smith 1987). The cardiac output was below the *in vivo* value (44 ml/min/kg, Houlihan et al., 1986), but comparable to those reported *in vitro* by Agnisola et al. (1994).

Linkage between cardiac output and coronary flow in the *in vitro O. vulgaris* heart was reported by Foti et al. (1985); during systole a proportion of the total cardiac output is collected in cut coronary veins. In our preparation, the amount of the aortic outputentering the coronary system ranged from about 17% (hypothermia) to 26% (Mix 45').These values are lower than those reported (e.g.: 41.5 ± 3.6% in Houlihan et al., 1987)using higher values of input and output pressures of 20 and 40 cmH2O, respectively. However, they are comparable to those obtained by Agnisola et al. (1989; 1994) where similar and more physiological loading pressures were used.

As expected from other *in vitro* studies where extrinsic neural inputs are removed, the baseline heart rate was lower than the *in vivo* range of 35-45bpm (Wells 1979; Wells1980; Wells and Smith 1987; Fiorito et al., 1998), but in the same range as those reported by other authors *in vitro*, i.e. 30 ± 0.7 bpm (Houlihan et al., 1987), 34 ± 1 bpm (Agnisola et al., 1989) and 26.3 ± 0.8 bpm (Agnisola et al., 1994).

Overall, with regards to baseline haemodynamic parameters (**Table 4**) there was little to differentiate the effect of various pre-treatments, with the exception of the hearts removed from animals anaesthetised with 3.5% $MgCl_2$ that produced a significantly higher afterload compared to hearts isolated under hypothermia and the significantly higher coronary flow in hearts isolated under $MgCl_2$ (1:1) and Mix 45' compared to hypothermia.

8.2 Frank-Starling curves

In terms of the responsiveness of cardiac performance (functional state of the heart as a pump in relation to contractility, work, and heart rate) to input pressures (Frank - Starling response), our preparation performed in a similar way to previous in vitro studies on octopus (Smith 1981; Foti et al., 1985; Houlihan et al., 1987); cardiac output has been shown to be primarily affected by changes in stroke volume, with input pressure having little effect on heart rate. Regarding the heart rate-preload relationship, it has been reported that in the in vitro spontaneously beating systemic heart of octopus the rate is directly affected by input pressure when the latter is above the physiological range as reported originally by Fredericq (1914), and later by Smith (Smith, 1981) and Foti et al. (1985). In contrast, the dependence of heart rate on the input pressure is irrelevant when physiological values are used (present study and Houlihan et al., 1987). In fact, here and irrespective of the anaesthetic pre-treatment, the heart rate did not change when preload increased.

As expected, increased preload produced an enhanced stroke volume and hence cardiac output; however, baseline conditions and the Frank-Starling response were only affected to a limited extent by different anaesthetic pre-treatments. In particular, systemic hearts removed from animals treated with hypothermia exhibited the best cardiac performance (as defined above). This is indicated by the maximum percentage increase in cardiac parameters (Q, SV, SW) achieved at the maximum input pressurereached and by the

number input pressure increments tolerated (**Fig. 4**). Specifically, hearts removed from animals exposed to $MgCl_2$ (1:1) or Mix 45' showed the worst cardiac performance compared to hypothermia, Mix 20', and $MgCl_2$ (**Fig. 4**).

Overall, on the basis of the Frank-Starling curves, the data suggest that magnesium chloride (1:1) and prolonged (45') exposure to Mix should be avoided as agents for procedures where recovery of the animal is required, because of the sustained residual effect on the heart and should not be used if "normal" cardiac tissue is required for *in vitro* or molecular studies. The mechanism underlying this residual effect is not known but in the case of Mix 45' this may be due to the extended time during which ventilation is markedly suppressed/absent leading to systemic hypoxia which is likely to damage the heart with its high aerobic metabolic requirement (Houlihan et al., 1987).

In contrast, Mix 20' and $MgCl_2$ (3.5% sw, 20min exposure) appear suitable formulations for anaesthesia in cephalopods where recovery is required.

8.3 Stress response

HSPs induction constitutes a ubiquitous mechanism which compensates for several stressful conditions (physical and chemical stress) (Snyder et al., 2001), with different animals exhibiting diverse sensitivity thresholds and tissue-specific expression patterns (Hofmann, 1999). HSP70 family was reported both in mammals and non-mammalian organisms such as fish (Gornati et al., 2004; Yamashita et al., 2004) and mollusks (Clegg et al., 1998; Gourdon et al., 2000; Piano et al., 2004, Boutet et al., 2003; Farcy et al., 2007). The constitutive HSPs are expressed under normal conditions and appear to be essential for protein folding or trafficking and regulated proteolysis in unstressed cells (Craig et al., 1983; Lindquist and Craig, 1988; Hightower, 1993). In contrast, inducible forms are over-expressed under stress conditions (Chapple et al., 1997).

Among the various signal transduction pathways involved in the responses to environmental stress, MAPKs have been shown to play a significant role (Schaeffer and Weber, 1999; Widmann et al., 1999; Kyriakis and Avruch, 2001). In particular, p38-MAPK has been characterised as the principal stress-kinase responsive to fluctuations in ambient osmolality and temperature (Zhang and Cohen, 1996; Kultz and Burg, 1998; Gon et al., 1998).

The results here obtained revealed that all magnesium formulations induced a stronger phosphorylation (hence activation) of both p38- and JNK-MAPKs in the cardiac tissue compared to hypothermia (**Fig. 6-7**). At the same time, these treatments induced a corresponding increase in HSP70, but not in the caspase-3, expression. The increased phosphorylation of MAPKs parallels the increased expression of HSP, supporting their cyto-protective role in anti-apoptotic cascade. Of note, as no significant differences in the HSP70 expression were revealed among the different treatments ($MgCl_2$, 1:1, Mix), a role of different osmolality in the observed effects may be excluded (**Fig.5**).

8.4 Anaesthetic concentration-response curves

The bradycardia induced by the various $MgCl_2$ formulation *in vitro* is consistent with reports that the heart rate is "very low" in *O. vulgaris* anesthetised with 3.5% $MgCl_2$in sea water (Fiorito et al., 2015) and "slow" in the same species anaesthetized with $MgCl_2$+EtOH (Grimaldi et al., 2013). Ethanol alone (from 0.125 to 1.0%) did not induce a significant bradycardia indicating that chronotropic effects of the $MgCl_2$+EtOH mixture (i.e. $MgCl_2$1.12 % + EtOH 1%) are not due to the ethanol. It should also be noted that the highest concentration of $MgCl_2$tested *in vitro* (2%) and which cause arrest in 50% of hearts was lower than the 3.5% commonly used for anaesthesia *in vivo*. This may indicate that *in vivo* additional mechanisms such as ganglionic or central nervous system reflexes operate to protect the myocardium.

In addition to the bradycardia, a significant reduction in stroke volume was observed with the effects being more marked with $MgCl_2$(1:1) and Mix compared to the same concentration (1%) of $MgCl_2$in sea water (**Fig. 8**). Again, comparison of the effects of Mix (1.12% + 1%) and ethanol alone (1%) shows that whilst there is a concentration related bradycardia with Mix (**Fig. 8**), this is not the case with ethanol alone, although both Mix and ethanol can produce cardiac arrest (**Fig. 9**).

It should be also noted that the highest concentration of ethanol we studied *in vitro* (1%) is lower than that commonly used (2-3%) to anaesthetize cephalopods when used as the sole agent (review in Fiorito et al., 2015).

8.5 Potential mechanisms involved in the cardiac effects of the $MgCl_2$ anaesthetic formulations

This study has established that three formulations of magnesium chloride used to anaesthetize cephalopods affect the cardiac function and in the case of $MgCl_2$(1:1) and Mix 45' we observed persistent effects. Investigation of mechanisms responsible was beyond the scope of this study but, for completeness, the most likely mechanisms are outlined below to provide pointers for future investigation.

8.5.1 Osmolality

We noted a difference in the osmolality of the magnesium chloride formulations (**Table 3-5**) used as anaesthetic agents in cephalopods: $MgCl_2$(sw) and Mix being hypertonic, and $MgCl_2$(1:1) hypotonic, compared to sea water.

Haemolymph in Mediterranean *O. vulgaris* has an osmolality of ~1300mOsm/Kg or higher (D'Aniello et al., 1986; Wells and Wells 1989), therefore Mix (1.12/1%) and 2% $MgCl_2$in sw are relatively hypertonic, while $MgCl_2$(1:1) is hypotonic, and 1% ethanol in sw is isotonic. Our data do not allow us assessment of the magnitude of any contribution of osmolality alone to the cardiac effects observed, as hypo- (e.g. 2% $MgCl_2$1:1), hyper- (e.g. 2% $MgCl_2$sw), and iso-tonic (e.g. Mix 0.56%/0.5%) $MgCl_2$formulations all produced a bradycardia and reduction in stroke volume.

Although *O. vulgaris* is reported to be intolerant to sea water dilution (Boletzky and Hanlon 1983; Vaz-Pires et al., 2004), a recent study showed no significant effect on water content of arm tissue from *O. vulgaris* exposed to a hypotonic solution (50% sea water osmolality) until 120 min (Amado et al., 2015) showing that dilution did not modify tissue hydration (swelling). This allows to exclude a key role of hyposmolality in the effects observed, at least for the time of exposure to anaesthetic formulations (immersion or perfusion) used by us. The same paper (Amado et al., 2015) reported that hyperosmotic solution (150% sea water osmolality) affected tissue hydration starting from 30min of exposure; this time is higher than that used here by immersion; at the same time, the hyperosmotic solutions used by perfusion ($MgCl_2$ 2% and Mix 1.12/1%) show an increased osmolality of about 8% respect to haemolimph, which appears too small to be considered crucial in the observed effects. In addition, changes in osmolality of plus or minus 20%

were without effect on transmission in the squid giant synapse (Bryant, 1958) providing a further indication that osmolality changes may not be primarily responsible for the biological effects of the various anaesthetic formulations used here.

Specific studies must be undertaken to test the effects of osmolality of the heart and in particular to ensure that the osmotic changes are not activating volume-regulated ion channels such as the TRPM3 member of the melastatin-like subfamily of the transient receptor potential (TRP) family activated by reduced extracellular osmolarity (Grimm et al.,2003). Osmosensitive channels have not been reported in cephalopods as far as we are aware, but studies in the mammalian heart showing chloride currents activated by cell swelling and inhibited by cell shrinkage (Duan et al., 2000; Huang et al., 2009) suggest that the osmolality of the solutions may have some role in mediating the changes and should be investigated.

8.5.2 Magnesium

The well known effects of elevated extracellular magnesium ion concentration in inducing bradycardia and cardiac arrest in mammals is consistent with the effects observed in this study. We estimate that the magnesium concentrations in the formulations are between ~2x (Mix 1.12% $MgCl_2$/1%EtOH) and ~4x (3.5% $MgCl_2$) those found in cephalopod haemolymph (D'Aniello et al., 1986; Brown and Lasek, 1990).

In mammals, cardiac effects of elevated magnesium ions are ascribed to depression of the sino-atrial node and atrio-ventricular conduction, caused by a direct effect of extracellular magnesium on transmembrane ionic current (Shine and Douglas, 1974; Specter et al., 1975) and/or an effect on sympathetic ganglia (Winkler et al., 1940; Engbaek, 1952; Dubé and Granry, 2003; Herroeder et al., 2011). In the octopus heart a putative pacemaker has been localized near the atrio-ventricular valves (Wells, 1983; Agnisola and Houlihan, 1994) and is a likely primary site at which $MgCl_2$ produces bradycardia in cephalopods.

Magnesium ions are a physiological blocker of Ca^{++} channels (Iseri and French, 1984). In the mollusc *Mercenaria mercenaria* extracellular hypermagnesia negatively affects cardiac rhythmicity in the due to an effect Ca^{++} (Devlin, 1993) providing further support for a major role of magnesium ions in mediating the cardiac effects of the formulations used in this study.

The anaesthetic solutions utilized in this study and commonly used in cephalopods also provide additional chloride ions. We cannot exclude that Cl^- currents may play aa role in the cardiac pacemaker activity.

In mammals, a Cl^- current appears activated by hyperpolarization and cell swelling, but inhibited by hypertonic cell shrinkage (Huang et al., 2009). During the cardiac action potential, the Cl^- current activation at negative membrane potential causes a depolarization (Cl^- efflux) of the resting membrane potential, while at membrane potential more positive than the Cl^- equilibrium potential it hastens repolarization (Cl^- influx). This mechanism may explain the results obtained with $MgCl_2$sw perfusion, since under hyperosmotic conditions a reduced Cl^- efflux may impair pacemaker function (Duan et al., 2000), but not those observed under almost isotonic conditions such as $MgCl_2$ (1:1) where its activation should be small, thus excluding the involvement of this anion current in the $MgCl_2$ (1:1)-dependent bradycardia.

8.5.3 Calcium, sodium, potassium and chloride

Cephalopod haemolymph has a relatively high concentration of calcium ions (in *O. vulgaris* 19 mEq/L according to D'Aniello et al., 1986) similar to sea water (approximately 10.5 mM/L according to Robertson 1953).

The 3.5% $MgCl_2$ (1:1) formulation has approximately half the concentration of Ca^{++}, Na^+, K^+ and Cl^- compared to sea water; this may account for the observed effects since myocardial contractility in cephalopods is very sensitive to extracellular [Ca^{++}] (Driedzic, 1985; Gesser et al., 1997) and Ca^{++} generates the cardiac action potential in bivalve molluscs (Devlin, 1993).

Extracellular [Ca^{++}] plays a pivotal role in both autorhythmicity and cardiac muscle contractility in molluscs (Hill and Yantorno, 1979; Driedzic, 1985; Devlin, 1993; Gesser et al., 1997). In octopus both extracellular [Ca^{++}] concentration (and thus transsarcolemmal Ca^{++}flux) and Ca^{++} release from the sarcoplasmic reticulum are crucial for systemic heart contraction (Gesser et al., 1997; Altimiras et al., 1999). Therefore, it is conceivable that the lower calcium concentration in the $MgCl_2$ (1:1) formulation may compromise cardiac function. In addition, the effect of lowered Ca^{++} concentration is probably exacerbated by the presence of a relatively high concentration of Mg^{++} which further antagonizes the effects of Ca^{++} (Iseri and French, 1984).

The persistence of the effects of exposure to $MgCl_2$ (1:1) when the heart is perfused with medium suggests an effect on intracellular regulatory mechanisms with a long duration of action although structural damage to the cardiac muscle and/or conducting system cannot be excluded.

The above hypotheses require direct testing with future studies also taking account of the role of taurine in modulation of the effects of calcium ions on the cephalopod heart as recently demonstrated in *S. officinalis* (MacCormack et al., 2016). Taurine is an important intracellular component present at high concentrations in marine molluscs, including *O. vulgaris* and it is involved in osmoregulation (Florkin and Bricteux-Grégoire, 1972) playing, together with inorganic ions and other organic solutes, a role in the volume regulation.
An increased extracellular osmolarity (like in $MgCl_2$ solution) may be partially matched by solute accumulation inside the cell. It was reported that *in vivo* taurine improves the activity of the sarcoplasmic Ca^{++} ATPase (SERCA) through phosphorylation of phospholamban; this enhances the rate of Ca^{++} handling by the sarcoplasmic reticulum increasing the calcium release for the next contraction (Shaffer et al., 2010). This mechanism may be inhibited in the case of $MgCl_2$ (1:1) where a putative loss of taurine may be accounted (iposmotic solution). However, this requires further studies since it has been reported that short-term cell volume regulation (30 min, similar to our time of exposure to anaesthetic treatment) is typically associated with changes in inorganic ion content and does not involve organic solutes (Gilles, 1987).
We can only speculate about the effects on the heart of simultaneously reduced K^+ and Cl^- in the 3.5% $MgCl_2$ (1:1) formulation, but we would expect that reducing extracellular K^+ would decrease excitability whereas reducing extracellular Cl^- would increase excitability.

The marked reduction in Na^+ concentration is likely to reduce the amplitude of the action potential in any neural tissue in the heart or ganglia based upon studies of theeffects of 50% reduction in Na^+ concentration on the squid giant axon (Hodgkin and Katz 1949).
Overall, whilst the effects of elevated magnesium ions on calcium fluxes in the heart provides the most likely explanation for the effects observed on stroke volume and heart rate, the contribution of osmolality and the altered concentrations of Ca^{++}, Na^+ ,K^+ and Cl^- acting in concert requires direct investigation.

8.6 Implications of the present study for anaesthesia in cephalopods

Magnesium chloride in various formulations has been extensively used to anaesthetize *O. vulgaris* and other cephalopods but few studies have been focused on its cardiovascular effects.

The use of the *in vitro* systemic heart of *O. vulgaris*, allowed us to demonstrate that different formulations of magnesium chloride utilized as anaesthetics cephalopods cause bradycardia in the isolated heart. This is consistent with the descriptive reports of heart rate in anaesthetised octopuses (e.g. Young 1971a; see also: Andrews et al., 2013; Fiorito et al., 2015). A marked fall in heart rate together with the effects of magnesium chloride formulations on stroke volume will reduce cardiac output leading to a decrease in brain perfusion. The fall in haemolymph flow to the brain together with the marked reduction of ventilation is likely to lead to ischaemia of the brain (and other tissues) that could contribute to the anaesthetic state induced by the variousmagnesium chloride formulations. Direct effects of the magnesium chloride formulations on the heart are likely to be compounded by the suppression of ventilation and muscular activity of the arms caused by anaesthesia which contribute to venous return (Wells 1983; King et al., 2005). In addition, acute hypoxia is itself associated with bradycardia and reduced aortic flow in cephalopods, as reported, for example, for *Nautilus pompilius* (Boutilier et al., 2000) and *O. vulgaris* (Wells 1983).

Furthermore, *in vivo* the anaesthetic formulations may also have effects on the brain vasomotor lobe or peripherally on the cardiac ganglia.

Despite the effects of magnesium chloride formulations on ventilation and the heart, following anaesthesia on returning an *O. vulgaris* to fresh sea water all externally assessed parameters return within ~30minutes (review in Fiorito et al., 2015).

Recovery from anaesthesia is usually uneventful with octopus feeding within an hour(for review see Fiorito et al., 2015). However, marked suppression of cardiac function is an undesirable property of an anaesthetic, particularly as in the case of the 3.5%$MgCl_2$(1:1 sw:dw) and prolonged exposure to $MgCl_2$ and ethanol mixture (Mix 45')where it is associated with residual deleterious cardiac effects.

In conclusion, this study has demonstrated for the first time the acute direct effects on cardiac function of three formulations of magnesium chloride used as anaesthetics in cephalopods following Messenger et al. (1985). A direct effect of Mg^{++} on cardiac calcium fluxes can account for the marked bradycardia observed in animals anaesthetized with $MgCl_2$. Evidence was obtained for a residual effect of $MgCl_2$on cardiac function that could compromise recovery, but this was apparent only with either prolonged exposure (45min) or using the sw:dw formulation.

Based upon the overall assessment of the acute (bradycardia/arrest and decreased stroke volume) and protracted (reduced Frank-Starling curves) effects, 3.5% $MgCl_2$in sea water and Mix formulations had the least deleterious combination of effects provided exposure time is minimized (within 30min or less).

These formulations may be suitable for procedures where relatively short duration (~20min) anaesthesia is required and for studies using non-invasive approaches (e.g. Grimaldi et al., 2007; Margheri et al., 2011).

In addition, these results pave the way for mechanistically oriented studies aimed to re-examine the most common anaesthetic practices used with cephalopods in relation to the application of Directive 2010/63/EU.

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