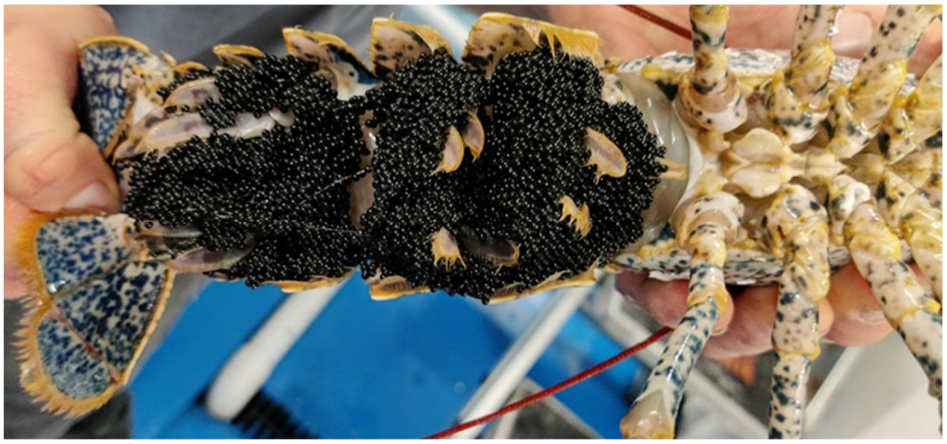
The lobster pregnancy test: a molecular tool in support of lobster fisheries and stocks in the UK

Lucy Sarah Irwin



A thesis for the qualification of: Master by Research

The University of Salford

2022

List of Contents

2. Materials and Methods

3. Results

4. Discussion

List of Figures

Figure 8a: Heatmap illustrating optical densities from a titration where lobster egg homogenate antigen and anti-lobster egg homogenate primary antibody IgG are serially diluted. A marked decrease in optical density is seen as both antigen and antibody dilution factors increase.

Figure 8b: Heatmap illustrating optical densities from a titration where lobster egg homogenate antigen and normal rabbit primary IgG antibody are serially diluted. While some unexpected peaks are observed (for example, 1:800 Ag with 1:400 Ab), the general trend of this optical density background shows a decrease in OD as Ab and Ag dilution decreases.

Figure 9: The overall difference in optical density from an ELISA where lobster egg homogenate antigen is targeted using anti-lobster egg homogenate primary antibody versus an ELISA where lobster egg homogenate is targeted using normal rabbit serum as primary antibody. There is an overall large and significant difference in optical density obtained from both variables.

Figure 10: Optical densities from 10 swabbed male specimens as a negative control. These results show an average optical density for males to be 0.0709, with the highest value obtained as 0.0915- seen as a potential anomaly. A negative cut-off value has been calculated from these values which can be applied in further tests.

Figure 11: Optical density values for six scrubbed individuals over time with a calculated negative cut-off value line applied. This data shows a downwards trend in optical density

over eight hours with a significant difference in optical density between the zero hours swab samples and the eight-hour samples.

Figure 12: Comparisons in optical density from assays run on lobster egg homogenate, average values for scrubbed females over 8 hours, males and normal rabbit sera targeting lobster egg homogenate antigen. The negative cut-off value line is applied. Both the egg homogenate and scrubbed assays show average values above the negative cut off value. Male swabs compared to using normal rabbit sera show they function well as a negative control.

Figure 13: Mean difference in optical density between hatching and non-hatching lobster eggs when an ELISA is carried out targeting lobster egg homogenate antigen with primary anti-lobster egg homogenate primary antibody. There is no significant difference in optical density between hatching and non-hatching eggs.

Figure 14: Both egg homogenate and haemolymph assay results for 10 berried individuals. The results show that while proteins can be detected in the haemolymph sample of berried females, the signal from egg homogenate is higher. Both haemolymph and egg values are above the negative cut-off value.

Figure 15: Frequency of optical densities from 50 swabs of females from a landing port where scrubbing status is unknown. The most frequent optical density value detected is 0.050-0.059. One sample was found to have an optical density above the negative cut-off value.

Acknowledgements

This study would not have been possible without the incredible wisdom, help and encouragement from my supervisors, Dr Ilaria Coscia and Dr Chiara Benvenuto. Their ideas, advice and creativity have inspired both this thesis and me personally. It has been an honour and pleasure to work with them.

I would also like to give thanks to Dr Antony Bodell, his vast knowledge in molecular biology has been invaluable to this study. Not only has he been a fantastic mentor to me, but he has also been a dear friend. Thanks also to Professor Michael Rogan, who's creative thinking and never-ending wisdom helped advance our molecular tools.

My supervisors and I would like to thank our fantastic collaborators; Dr Carly Daniels and Jake Scolding MSc at the National Lobster Hatchery for providing us with the samples we needed for the study and their great ideas and engagement with the project. We would also like to thank Collin Trundle at the Inshore Fisheries Conservation Authority in Cornwall for his guidance and help in sampling, as well as bringing new ideas into the project. We would also like to thank Chris Graham and the Marine Management Organisation for his experience and guidance.

Our gratitude goes to the project funders, without whom this study would not be possible: The Marine Management Organisation, IFCAs and The University of Salford.

Thanks also to all my wonderful supportive friends and family for supporting me throughout the study and encouraging me to keep going at the toughest times. I could not ask for a better support network.

Declaration

I hereby declare that the work in this thesis is my own, unless otherwise stated.

List of abbreviations

CL- Carapace Length

ELISA- Enzyme-linked immunosorbent assay

KDa- Kilodaltons

CPUE- Catch per unit effort

MPA- Marine Protected Area

NTZ- No Take Zone

OD- Optical Density

SDS-PAGE- Sodium dodecyl sulphate polyacrylamide gel electrophoresis

NLH- National Lobster Hatchery

EDTA- Ethylenediaminetetraacetic acid

NaCl- Sodium Chloride

PBS- Phosphate-buffered saline

BSA- Bovine Serum Albumin

BCB- Carbonate-bicarbonate buffer

Abstract

The European Lobster (*Homarus gammarus*) is one of the highest value shellfish species landed in the UK and accounts for a large part of British shellfish fisheries. However, high demand for this sought-after commodity has led to overfishing and declining stocks in the last few decades forcing local authorities to impose a course of strategies to conserve stocks. Catch size limits, v-notching sexually mature individuals and a ban on landing ovigerous (egg bearing) females have all been introduced. Intelligence is showing however, that a practice called 'scrubbing', where eggs are removed from the abdomen of female lobsters to appear non-ovigerous is taking place. The subsequent harvesting of these individuals will cause further decline in stocks, due to the prevention of stock replenishment. This study has produced a molecular tool for detecting this practice by using indirect ELISA to target antigens exposed in the breaking of the eggs (left behind in a traceable layer on the abdomen). In addition, this study answers further research questions such as the potential for protein degradation over time leading to false negatives. It also suggests future work for fortification of this molecular tool so it could potentially be implemented into a rapid, cost-effective lateral flow test. Ultimately, this would provide fisheries protection services the means to enforce these important laws in the field.

1. Introduction

Shellfish represent 20% of overall seafood landings in the UK , and the highest overall value compared to demersal and pelagic fish, with an estimated catch total of £331 million in 2021 (up from £262 million in 2022) (House of Commons Library, 2022). Within the shellfish industry, *Homarus gammarus* (European Lobster) is one of the most exclusive and valuable commercial crustaceans, with a recent estimated value of £16,000 per tonne, far exceeding commercially caught crab species (Hinchcliffe *et al.*, 2022). Attributed to this high demand and value, there are several fisheries management strategies in place to conserve what is an overexploited wild stock, (landing sizes are increasing year on year from seemingly smaller area sizes) (Cefas 2020). These include minimum landing sizes (length of carapace on individual), v-nothching sexually mature individuals, catch size limits and a ban on landing ovigerous (egg bearing) lobsters (Hinchcliffe *et al.,* 2022).

1.1 Homarus gammarus

The European lobster, *Homarus gammarus* (Linnaeus, 1758) and American lobster, *Homarus americanus* (H. Milne Edwards, 1837) are the only extant representatives of the *Homarus* genus within the decapod family, Nephropiodae (De Grave, *et al*., 2009). While morphologically very similar, there is a large discrepancy in how well studied *H. gammarus* is compared to *H. americanus,* largely because *H. americanus* is far more abundantly landed (Ellis *et al.,* 2015; Phillips, 2013). *H. gammarus* can be distinguished from *H. americanus* by its colour and absence of ventral tooth on the rostrum as well as spatial distribution but are otherwise difficult to distinguish (Jørstad et al., 2007). Distributed in proximity to British shores, *H. gammarus* is the most commonly landed lobster species in the UK (the other being spiny lobsters, *Palinurus elephas* and *Palinurus mauritanicus*)

(Hinchcliffe *et al.*, 2022). The high commercial value of *H. gammarus* is generally attributed to the large-bodied nature of the species (Phillips, 2013).

1.2 Purpose of the study

A high volume of *H. gammarus* is landed in the UK, with a general increase in overall catch size in the past decade (Cefas, 2020). Concerns around stock depletion have led to the implementation of a statutory instrument in England, which prohibits the taking of ovigerous (egg bearing, or berried) females (The Lobsters and Crawfish, Prohibition of Fishing and Landing (England) Order, 2017). The intended outcome of this instrument means sexually mature female lobsters can replenish stocks by hatching eggs and continuing to reproduce.

The introduction of this law in English waters has instigated growing concerns amongst the Inshore Fisheries Conservation Authorities (IFCAs) and the Marine Management Organisation (MMO) about the removal of eggs from ovigerous females (and subsequent landing) to pass visual inspections from fisheries and conservation officers. This is a process known as 'scrubbing' or 'washing' and involves the removal of eggs using a stiff yard brush or hose to forcibly remove the eggs from the abdomen of the lobster (Marine Management Organisation, 2018). This illegal practice is seemingly growing in scale with increased concerns about stock depletion from the landing of ovigerous lobsters from fishers and authorities alike (Badger, 2017). Prosecutions have been made to combat scrubbing and landing of breeding females, most recently in January 2023 where a fishing vessel in Cornwall was fined £20,000 after it was found to be carrying berried females, scrubbed females (visually appeared to have undergone rough treatment) and scrubbing brushes

(The Fishing Daily, 2020). This study investigates the use of molecular tools to produce a more forensic approach to detecting scrubbing.

1.3 *Homarus gammarus* distribution

H. gammarus is distributed in a northern range, occurring from northern Norway to south-eastern Sweden and Denmark (absent from the Baltic Sea, thought to be due to salinity and temperature extremes). It is also widely distributed along the coasts of mainland Europe, Britain and Ireland down to the Atlantic coast of Morocco (Prodöhl *et al*., 2006). Distribution can vary seasonally, with more utilisation of individual home ranges in spring than during autumn (more space is covered by males than females) (Skerritt *et a*l., 2015).

1.4 *Homarus gammarus*: reproductive biology

The size at sexual maturity for *H. gammarus* can be variable and rely on a range of parameters, for example sexually mature females are found to have a smaller Carapace Length (CL) than the average of 80-140mm in the North Sea than other members of the species elsewhere (Phillips 2013). Also, it may be difficult to determine sexual maturity as, for example, a female may be physiologically capable of producing a viable clutch of eggs long before it is visible on the abdomen (Lizarraga-Cubedo, 2003). Generally, *H. gammarus* mate and spawn in late summer, with hatching season beginning from late May to August (peaking in July with water temprature at 16°C) the following year (Schmalenbach & Franke, 2010).

The reproductive parts of *H. gammarus'* anatomy are located in or on the abdomen (figure 1a and 1b), the last of three segments after the head and thorax (all of which hold two appendages each). There are six sets of pleopods (swimmerets), the first of which are

sexually dimorphic (where gonopods are found in males) (Factor, 1995). Conversely, females have gonopores and oviducts which are connected to a H-shaped set of parallel lobed ovaries (anterior and posterior) with a seminal receptacle functioning as sperm storage (figure 1b) (Erkan and Ayun, 2014).

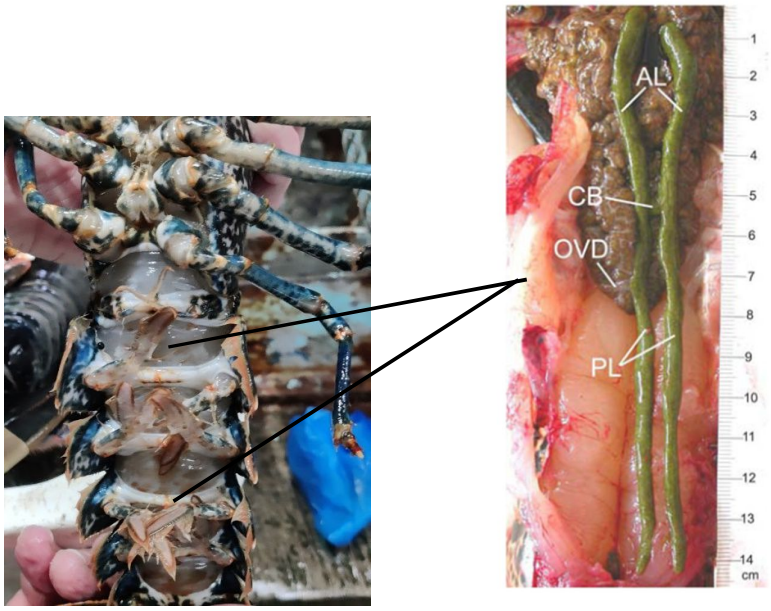


Figure 1. A. Abdomen section of *Homarus gammarus* (Photograph by Lucy Irwin, 2021). *B. Homarus gammarus female reproductive system. AL: Anterior Lobes, PL: Posterior Lobes, OVD: Oviducts, CB: Central Bridge (Erkan and Ayun, 2014)*

H. gammarus fecundity and size at sexual maturity is based on age determination from carapace lengths (CL). The average CL for sexually mature lobsters is between 80 and 140mm, clutch (the term denoting the brood of eggs on the abdomen) size increases with CL however variations also occur with temperature and location (Tully, Roantree, & Robinson, 2001; Hinchcliffe *et al.*, 2022). Further, it has been suggested that larval quality

can be influenced by egg characteristics in decapods, as energy content increases with female size (Sibert, Ouellet & Brêthes, 2004; Attard & Hudon, 1987).

1.5 Yolk and egg composition

Contents of egg casings mainly comprise of proteins, free amino acids, glucids and lipids which vary in concentration over the course of their development (Mellinger, 1995). *H. americanus* exhibits two stages of vitellogenesis in the formation of egg yolks whereby primary glycoprotein is produced in the first stage and a lipoprotein complex (present in haemolymph and secreted into eggs) is formed (Dehn, Aiken, & Waddy, 1983). Studies of amino acid and lipid content during egg development in *H. gammarus* show a decrease in both essential amino acids, such as lysine and leucine, and major nonessential amino acids (glutamic acid, aspartic acid, valine and glycine). In contrast, free amino acids increase during egg development. Lipids such as monoacylglycerols, triacylglycerols, sterols and diacylglycerols decrease (Rosa *et al*., 2005). Studies on yolk deposition in *H. americanus* have confirmed the presence of vitellin (common yolk protein), with binding assays showing maximal binding activity from binding sites within solubilised membranes taking place at the beginning of vitellogenesis, when targeting vitellin receptors, and decreasing in older oocytes (eggs) (Laverdure, & Soyez, 1988; Tsukimura, 2001; Tui et al., 2009). Vitellin (Vn) was analysed by gel filtration and was found to have a molecular mass of 360 kDa while such protein was confirmed by an enzyme-linked immunosorbent assay to not be present in the haemolymph of male lobsters (Tsukimura *et al*., 2002). It was also found that Vn was formed from vitellogenin (Vg) after uptake from the ovaries and a 40-fold increase in Vg levels is seen during the reproductive cycle of adult females (Tsukimura *et al*., 2002). Another study found multiple Vg genes as a result of genomic cloning where the

hepatopancreas *HaVg1* had low levels which subsequently increased as oocytes matured (Tui, 2009). Little else is known about the exact protein structure of *Homarus* egg yolks.

1.6 Hatching and larval stages

During pregnancy, *funiculi* (attachment stalks) hold eggs onto the pleopods in clutches, facilitated by a substance called *cement* and non-plumose setal hairs (figure 2). Eggs are held on the abdomen for a duration of 9-12 months, where they develop in four stages before hatching into a fifth larval stage (Vaughan, 2008). Eggs are categorised into these stages based on characteristics such as the size of the prominent eyespot and colouration (Johnson, Goldstein, and Watson, 2011). During the first stage, eggs have an average diameter of 1.8mm, with a dark green colouration and a round to oval shape, by stage two eggs are more oval in shape, are green/blue in colouration and a visible eyespot. The dark green colouration begins to change to red in stage three as yolk begins to be absorbed and chromatophores develop, the embryo is also formed and visible with eyespots (figure 3). At stage four, eggs are a diameter of 3.0mm, the embryo fills the egg casing and is visibly developed (Pandian, 1970; Hinchcliffe *et al.*, 2022).

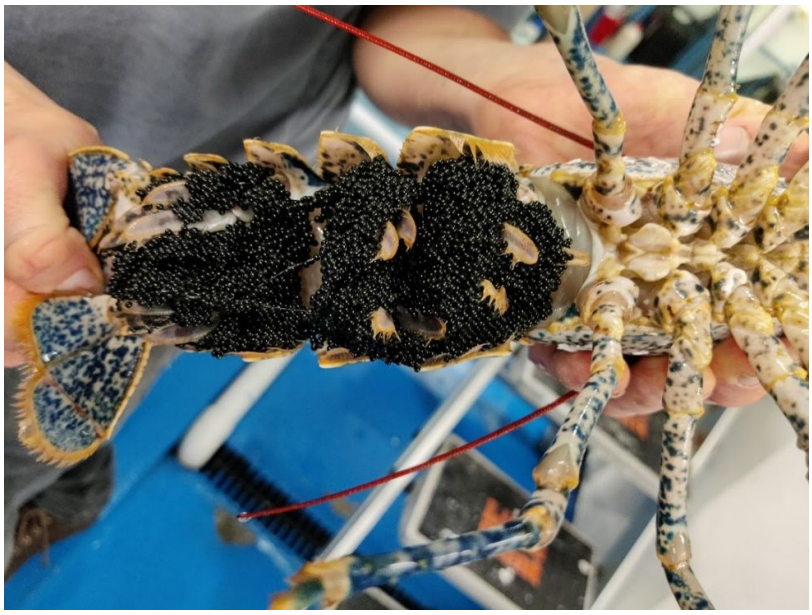


Figure 2. Berried (ovigerous) lobster with clutches of eggs on abdomen (Irwin, 2021)

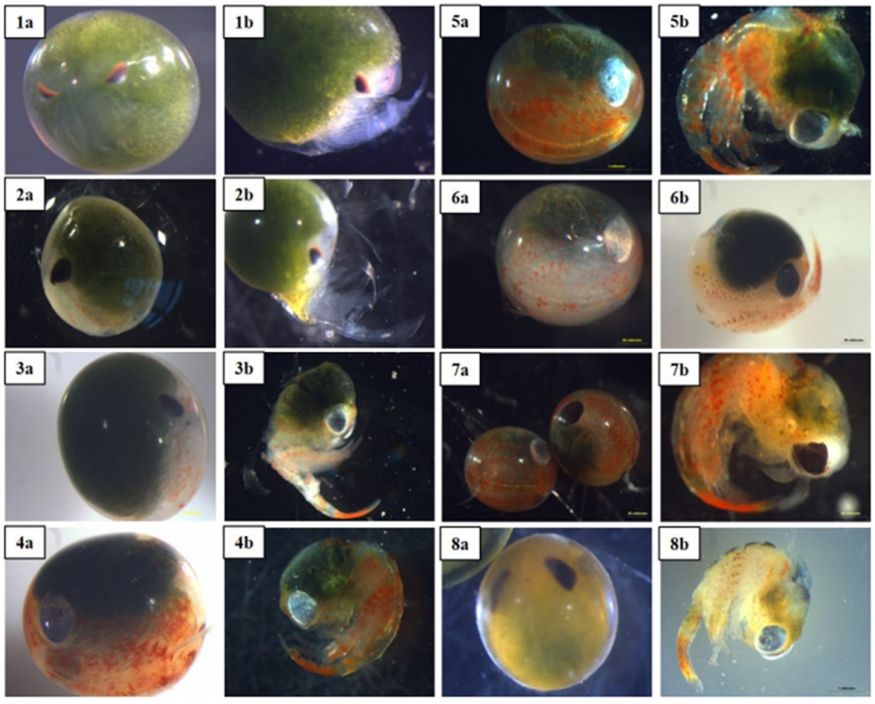


Figure 3 European lobster embryos at all stages of development. Increasing yolk consumption and changes to the shape of the egg from 1a through to 8b where the embryo begins forming into a juvenile (Emre, ACARLI & Berber, 2020).

Part of the clutch hatch as 'pre-larva' in the initial stages of the 2-3 week hatching period where larvae remain attached to the pleopods (Rotzer & Haug, 2015). The tail of the ovigerous female then extends and rapid movement of the pleopods occurs to expel larvae, causing pre-larvae to moult into stage one of the three pelagic larval stages (Hinchcliffe *et al.*, 2022). Typical of Nephropidae, three zoea stages are exhibited and one subsequent megalopa stage, each succussing the other by a moulting (these stages are more abbreviated in *H. gammarus* than in *H. americanus* in terms of how drastic metamorphism is (Rotzer & Haug, 2015). The larval period lasts for around 12 days (with an optimal temperature of 20-22°c) or up to three weeks. After this, chemical queues result in larvae moving to a benthic dwelling, close to the area of spawning where suitable settlement substrate will be found- this is the fourth stage juvenility (Gerlach & Artema, 2012).

1.7 Larval survivability, the role of hatcheries

One study has have shown decreases in fecundity due to temperature increases from climate change and anthropogenic emissions of carbon dioxide leading to a reduction in surface pH (Rato *et al*., 2017). The survivability of pelagic larvae is subject to physiological factors such as changes in pH, salinity, pollution, nutrition (or lack thereof) and temperature. It is estimated that only 0.005% of hatching lobsters survive the first three post-hatch stages to become benthic at stage 4 (Vives i Batlle *et al.,* 2010). Hence, lobster hatcheries (of which there are ten in the UK) have emerged as a working solution to increase outcomes for hatching broods (Hinchcliffe *et al.,* 2021).

The primary function of hatcheries is to use increase wild populations and stocks to support fisheries by releasing aquaculture-hatched juveniles, cultured until stage 4 of the larval cycle (where they are considered to have higher survivability (Hinchcliffe *et al*., 2022). This

stock replenishment has been shown to contribute to commercial fishery landings, as one study identified hatchery-reared juveniles in a study using a micro-tag system and another confirming augmented recruitment, but highlighting this restocking alters the genetic structures of groups in different geographical areas (Bannister, Addison, & Lovewell, 1994; Jenkins *et al.,* 2020).

1.8 Lobster fisheries in the UK

H. gammarus is a highly important in terms of economic value, with a current worth of ca. €16,000 per tonne, equating to €40 million per year in terms of market value in European countries from a landing mass of around 5,000 tonnes across the species range (Hinchcliffe *et al.,* 2022). Landing of European lobster in England and Wales show a general increase in the amount of lobster being landed between 2013 and 2018 but a decrease in area from which they are being caught (Cefas, 2020). Overfishing has led to large declines in lobster stocks in the last few decades (Carere *et al.,* 2015). It has been argued that technological creep, advances in landing technologies over past decades, has masked real landing trends and decline in stocks based on fishing data from Norway. Here, the Catch-Per-Unit-Effort (CPUE) index has suggested an 8% increase in lobster abundance, however when technological creep is taken into account, the reality shows a 57% decline, this may mean that technological creep elsewhere is masking the already visible decline of stocks across the UK (Kleiven et al., 2022). Attributed partially to the non-migratory nature of the species, *H. gammarus* has benefited from the use of marine protected areas (MPAs) and No Take Zones (NTZ) as an effort to conserve stocks. A mark-recapture study in Sweden found that in one MPA where fishing for European lobster was banned for 14 years, found positive trends in both mean body size and CPUE, indicating an increase in abundance. A decrease

in sex-specific natural mortality was also seen (Moland *et al*., 2013). Further, another study has shown that MPAs along the Norwegian Skagerrak coast increased CPUE by 245% and mean size of lobster by 13% for *H. gammarus* compared to an 87% increase in a control area and negligible mean size change (Moland *et al*., 2021). In terms of the effect MPAs have on genetic diversity, one study on *H. gammarus* cites high levels of connectivity between MPAs and non-MPAs and a lack of fishery induced genetic erosion as reasoning for high levels of genetic variability and lack of genetic bottlenecks (Watson *et al.,* 2016). Recent examples of the success of NTZs have been reported in the UK as well, lobster populations off the Scottish coast have been shown to be more abundant and have a higher average CL in an NTZ in the Firth of Clyde than in fished areas outside the zone (Crimmins, 2018).

As well as regulations about where fishing for lobster can occur, other strategies are in place for fisheries management such as v-notching; maximum sustainable yields, catch size limits, and prohibitions on landing berried (ovigerous) lobsters (Nicosia & Lavalli, 1999). V-notching is a scheme that has been implemented both in the UK and abroad (Acheson, & Gardner, 2011). In the interest of conserving a reproductive population within stocks, the aim is to remove a v-shaped section of the exoskeleton from the Uropod (inner tail flap) to mark individuals of reproductive size (87mm CL length) as 'breeding', meaning it cannot be removed from the stocks (figure 4). While in some fisheries this scheme is voluntary amongst vessels, fishery conservation authorities in the UK have written v-notching into byelaw (NIFCA, 2017). A study on the impact of v-notching showed no significant difference in survivorship between v-notched and control lobsters but did indicate a decline in retention of notches after moults (DeAngelis *et al.,* 2010). At around 85mm CL length, moulting becomes annual, meaning it is approximated that v-notching gives female

lobsters a maximum of two breeding cycles to release their eggs, replenishing stocks (NIFCA, 2017).

Figure 4. V-Shaped notch on the tail of a sexually mature female lobster (Goetting & Whiffen, 2011).

Due to overfishing of the European Lobster off English shores, with evidence that the size of the spawning stock was no longer sustainable, a Statutory instrument was introduced in 2017 called 'The Lobsters and Crawfish, Prohibition of Fishing and Landing (England) Order 2017' which effectively banned the landing of ovigerous ('berried') females into English ports, regardless of catch origin. There is intelligence however, to suggest some vessels may be evading this legislation by removing eggs from ovigerous females with a brush or high-pressure hose- a process known as scrubbing, so lobsters do not appear berried (Vaughan, 2008; MMO and IFCAs *pers*. *comms*). An example of this occurred whilst this legislation was only regionally enforced in 2006 where one fisherman plead guilty to removing six ovigerous females from stocks, eleven fisheries officers and two joint

committee patrol vessels were needed to bring the case to court, where the defendant was fined £2.700 for illegal fishing (Vaughan, 2008).

1.9 Detecting scrubbing

Given the legal prohibition of landing berried lobsters, it has become crucial to be able to identify when illegal scrubbing has been carried out before the individual lobsters enter the supply chain. Several methods have been trialled. Primary tests are visual, microscope analysis of pleopods on scrubbed specimens has found damaged egg sacs and snapped setal hairs, compared to naturally spent females where egg casings remain and are emptied as opposed to smashed (Vaughan, 2008). Other visual cues can include redness on the abdomen of the female; however, this cannot in itself be used as evidence for scrubbing.

In 1973, a test was developed to identify evidence of scrubbing by detecting residual cement (the substance that bonds eggs to nonpulmose setal hairs) by using a haematoxylin dye on pleopods (Karlsson & Sisson, 1973). This involves the removal of pleopods, which does not affect the survival of the lobster (Vaughan, 2008). It is widely used in the USA but has only gone as far as being replicated by UK fishery authorities to test reliability, which has shown it to be highly reliable where traces of evidence do not easily degrade over time (Vaughan, 2008).

Since the implementation of this test in the US, it emerged that strategies to removed eggs has developed to evade detection. A dilute bleach solution was found to be used to remove eggs. Hence, a chlorine bleach detection test was proposed whereby cotton swabs were dipped in starch-iodine solution, which turned blue when rubbed on the abdomen of

bleach-dipped lobsters. This is a non-invasive method of testing which produced no false positives(Heckman *et al.,* 2000). Another study using starch-iodine to detect traces of bleach on illegally caught spiny lobsters however, found that the average amount of time the bleach was detectable for was 6.2 and 9 hours in the laboratory and field, respectively (Wilson *et al.,* 2008). This indicates a depletion of evidence within a relatively short window, meaning enforcement officers would have to act quickly to prosecute illegal practices. Thus, enforcement officers are left with a limited set of tools to detect the illegal landing of scrubbed lobsters.

1.10 Enzyme Linked Immunosorbent Assay (ELISA)

Often, traces of proteins can be detected using an Enzyme-linked immunosorbent assay (ELISA). The ELISA is a widely used test in many industries including environment, food and healthcare. The main purpose of the assay is to target analytes, most commonly antigens or antibodies, using antigen-antibody complexes (Karim 2018). Developed in the 1960's for disease detection, it allows for the 'detection and quantification of specific protein within a complex mixture' (Karim 2018). Multiple different types of ELISA have been developed, all with the main principle of targeting antigen or antibody with corresponding antibody or antigen, which subsequently interacts with an enzyme which reacts with substrate, causing a colour change from which an optical density reading can be obtained. Antigen or antibody is passively absorbed into a 96-well microtiter plate, which allows for an indirect, direct, sandwich or competitive ELISA to be performed (Crowther, 2008).

In an indirect ELISA, antigen sample is diluted in a binding agent and used to coat the wells of a microtiter (96 well) plate. After a binding period and washing stage, a primary antibody, specific to the antigen being targeted, is introduced to the wells, and left to bind

over a fixed time interval. A secondary antibody, complementary in shape to the animal the primary antibody is raised in, is inserted into the wells. The secondary antibody is conjugated to an enzyme that will react with an introduced substrate and invoke a colour change if all bounding stages have occurred (figure 5) (Thermofisher Scientific©, 2022). The intensity of this colour change can be measured by absorbance in a plate reader and the optical density recorded (Karim 2018).

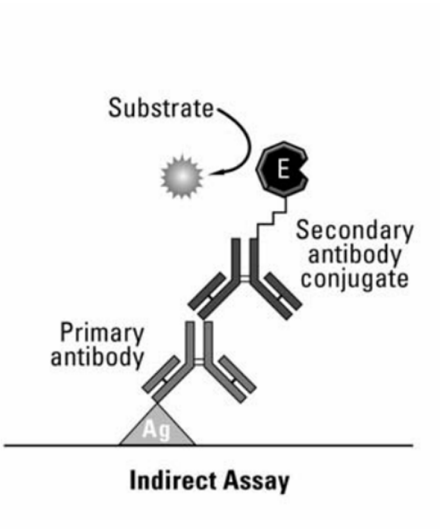


Figure 5. Indirect ELISA. Antigen is bound to the well of the microtiter plate, primary antibody is complementary to the antigen, allowing binding. Subsequent secondary antibody (anti-primary antibody) attaches and is conjugated to an enzyme. This enzyme evokes colour change is all binging has occurred, when a substrate is introduced (Thermofisher Scientific© 2022)

1.11 SDS-PAGE and Western Blot

SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) is a technique commonly used to separate proteins by size. Sodium dodecyl sulphate is used as an anionic detergent which binds to proteins and causes the polypeptides to denature creating a negative charge. When an electric current is then applied to the proteins in a tank, the proteins migrate towards a positively charged anode, proportional to molecular weight

(ranging between 10-200kDa) (Osborne & Brooks, 2006). Proteins travel in bands on a gel and can be detected using staining and Western Blot (Field & Ream, 1998). Western blotting allows for the electro transfer of proteins to a solid supporting membrane. This allows for further information to be obtained about the protein, such as reactivity with specific antibodies, aiding characterisation of the protein sample (Osborne & Brooks, 2006).

1.12 Aims

The main aim of this project is to develop and optimise an ELISA-based test targeting the eggs protein putatively left behind on the shell by mechanical scrubbing. The project also aims at estimating the protein decay time, in order to provide enforcement officers with a reliable, fast and portable tool that could quickly and reliably detect scrubbed individuals, by just swabbing the underside of the carapace, where eggs are usually carried.

All egg samples analysed has been obtained under special dispensation in collaboration with the National Lobster Hatchery, following ethical approval. Proteins detected have been targeted with externally manufactured anti-lobster egg homogenate primary antibody, raised in rabbits. If lobster egg homogenate protein is detectable in the sample, a secondary anti-rabbit, enzyme-conjugated antibody will bind to the primary antibody and cause a yellow colour change upon the addition of a substrate- quantified using an optical density plate reader. In establishing positive and negative controls in the form of scrubbed females and male lobsters respectively, a negative cut-off value was calculated for application in the testing of unknown specimens. The study also investigates protein degradation over time in different conditions (potentially leading to false negatives) as well as determining whether pregnancy stage effects protein activity. This study provides the

foundations for a rapid, low cost and non-invasive swab test which can be implemented by fisheries officers in the UK in the enforcement of prohibition of landing ovigerous lobster laws, hence aiding the sustainable management of this important resource.

2. Materials and Methods

2.1 Sample Collection and processing

Polyclonal antibodies (anti-lobster antibody raised in rabbits) were produced by ThermoFisher Scientific™ (Rockford IL, USA), using lobster egg homogenate. Antibodies were produced by preparing a homogenisation buffer using 1x PBS (phosphate buffered saline) with 0.1M NaCl (sodium chloride), 1mM EDTA (Ethylenediaminetetraacetic acid), 0.1% Tween-20, and 1X protease inhibitor. Eggs were placed in a dish with homogenisation buffer and mechanical means used to facilitate homogenisation. The resulting homogenate was then dialysed into 1x PBS (Phosphate-buffered Saline) from immunisation, and a protein quantification assay to determine concentration and yield was carried out. Primary anti-lobster egg homogenate antibody was extracted from the rabbit over several weeks, antibody used in all assays during this study were taken from the extraction at 72 days.

Research collaborators at the National Lobster Hatchery (NLH) in Padstow (Cornwall) provided a range of egg samples from 12 egg-bearing individual lobsters held in their facility. Parameters such as egg colouration and development stage were noted. Small clusters of eggs were removed from the clutch and placed in 1.5ml Eppendorf tubes containing 0.5ml of 1% PBS solution (Oxoid™, Thermo Fisher Scientific™, UK) and subsequently stored at -20°c.

Eggs, abdominal swabs, and haemolymph samples were initially used for the indirect ELISA. Firstly, homogenised eggs were used to assess the level of reactivity of the protein mixture towards anti-lobster primary antibody. Once positive results from eggs were determined, haemolymph and swab samples were taken from multiple live specimens. This was done by inserting a needle attached to a 1ml syringe into the softest part of the abdomen and

withdrawing a haemolymph sample. The specimen was returned to water as soon as this was complete to prevent any further blood loss or distress.

Swab samples were taken from three different types of individuals: scrubbed females from the hatchery, males, and unknown females from a fishing merchant. In all cases, foam swabs (Akaigu, UK) were used to make contact with the bases and corners of each segment of the abdomen including pleopods. The same technique was used for field testing at the landing port, located in Newlyn in Cornwall. Swab samples were kept at 4°c for 48 hours until they were able to be returned to the lab where they were kept at -20°c until use.

Swabs were then placed in 2ml collection tubes containing 1ml PBS. Foam tips remained submerged in the PBS, and kept frozen at -20°c. In preparation of sampling, six early-stage berried females held in tanks at the NLH facility had all eggs scrubbed using a stiff-bristled brush, the specimen was then rinsed with sea water. Scrubbed females were swabbed immediately after scrubbing, then stored in tanks with two subsequent swabs after 4 and 8 hours. Eggs were weighed out at 0.5g per individual and homogenised in 300µl of PBS, passed through a 1ml syringe with a 24x gauge needle attachment 5 times until a liquid consistency was achieved. This crude mixture was then used to bind to the wells of a HB4 Immulon flat bottom high binding plate (Thermofisher™, Waltham, MA USA).

Test were run to determine whether lobster egg homogenate antigen degraded over time in both wet and dry conditions. This was done by aliquoting 5µl of crude egg homogenate sample onto a series of glass microscopy slides. For wet conditions, these slides were immediately placed in a petri dish containing 10ml of artificial seawater (Instant Ocean, Blacksburg, VA, USA) to point of submersion. Slides were swabbed and placed in a 2ml tube containing 1ml of PBS and immediately frozen at -80° to capture the protein degradation

point at the time. The first swab was taken immediately after egg homogenate was applied to the slide and then 15-minute intervals thereafter for a duration of four hours.

ELISAs

2.2a Bradford assay

A Bradford protein assay was performed to obtain an estimate for protein concentration. A series of dilutions of 5mg/ml BSA (Bovine Serum Albumin) (Fisher BioReagents™, Waltham, MA, USA) (solution was produced. During each dilution, each tube was vortexed and left to equilibrate for 1 minute. Concentrations of neat, 1:4 and 1:9 were made for unknown samples (lobster egg homogenate) by diluting in dH_2O and vortexing for 1 minute. A HB4 96 well microtiter plate was loaded with 40µl of each standard dilution and unknown sample in duplicate. BioRad stain (Biorad, Watford, UK) was loaded into each well at 200µl as well as 2 blank wells. The optical density was recorded using a Byony Absorbance 96 portable plate reader (Enzo Life Sciences, Exeter, UK) at a wavelength of 450nm.

2.2b Checkerboard titration

A checkerboard titration was carried out to optimise the assay by coating a plate in a series of dilutions and running an assay to determine the best dilution to used based on optimum optical density (Voller *et al*, 1976). All wells in rows 2-8 were loaded with 100µl of BCB (Carbonate-bicarbonate buffer) (Merck Life Science UK Limited, Gillingham). Egg homogenate sample was diluted in BCB at 1:100 and 200µl loaded into column 1 of the plate, 100µl was then take up and loaded into column 2. This was repeated across the plate and the final 100µl discarded, producing a series of dilutions from 1:100 to 1:20,480. As

per the ELISA protocol (described in section 2.2c indirect ELISA), the plate was incubated at 4°c overnight. After this, the plate was washed by aliquoting 100µl of 0.1% PBS with Tween 20 (both Merck Life Science UK Limited, Gillingham) into all wells, this was left for one minute before being emptied and the process repeated thrice. Wells were then blocked with 0.3% PBS Tween 20 with milk (Merck Life Science UK Limited, Gillingham) by loading 200µl into each well except blanks and incubating at 37°c for 30 minutes. This process reduced background noise- blocking out interference from unbound antigen. After another three washing steps as previously described, primary antibody (anti-lobster egg homogenate raised in rabbit) was then diluted in blocking buffer (0.3% PBS Tween 20 with skimmed milk) at 1:100 and loaded (100µl per well) into the plate in a serial dilution from row A to H (in the same manner as the previous serial dilution but horizontally). The plate was incubated at 37°c for a further 30 minutes, then washed. One hundred microlitres of secondary anti-rabbit antibody was then loaded into all active wells (except relevant controls and blanks) at a dilution of 1:16,000 in 0.3% PBS Tween 20 with no milk). The plate was incubated again at 37° for 30 minutes and washed. A substrate solution of 5mg/ml p-nitrophenylphosphate (Thermo Scientific™ Waltham, MA, USA), 1M diethanolamine and 0.5mM MgCl2 pH 9.8 (Thermo Scientific™ Waltham, MA, USA) was loaded into all wells at 100µl, and the optical density measured using a Byony Absorbance 96 portable plate reader at a wavelength of 450nm.

2.2c Indirect ELISA

Indirect ELISA (enzyme-linked immunosorbent assay) was performed as described by Lin (2015) whereby antigen samples are bound to a 96-well microtiter plate through passive absorption. A working assay was optimised for both antigen coating and primary antibody

concentrations using a checkerboard titration described by Voller *et al* (1976) where a dilution for both antigen and antibody was determined as 1:400.

For each assay, a 96-well microtiter plate was coated with 100µl of each sample diluted at 1:400 in carbonate-bicarbonate buffer (pH 9.8) in vertical duplicate. Plates were left covered and incubated overnight at 4°c. Subsequently, plates were washed to remove any unbound or excess antigen by loading the wells with 100µl of with 0.1% PBS Tween 20 and leaving for 1 minute (repeated 3 times). The same washing process was used throughout each assay. All antigen-coated and control wells were then loaded with 200µl of blocking buffer (0.3% PBS with Tween 20 and skimmed milk powder).

Plates were covered and incubated for 30 minutes at 37°c, then washed. One hundred microliters of primary anti-lobster antibody, diluted in 0.3% PBS Tween 20 and skimmed milk at 1:400, was loaded into each antigen-coated well and incubated for a further 30 minutes at 37°c. Plates were washed and wells were coated with 100µl of secondary antibody conjugated to alkaline phosphatase and diluted at 1:16000 in 0.3% PBS Tween 20. Plates were incubated once more at 37° for 30 minutes and washed. A substrate solution of 5mg/ml p-nitrophenylphosphate, 1M diethanolamine and 0.5mM MgCl2 pH 9.8 was loaded into all wells at 100µl and optical density measured using a Byony Absorbance 96 portable plate reader at a wavelength of 450nm.

2.3 SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis)

A crude antigen sample was produced by crushing eggs using a pestle and mortar. Half the crude extract was centrifuged at 13,000 rpm for 5 minutes. The crude and centrifuged supernatant samples were mixed with 2X Laemmli reducing buffer (0.125 M Tris HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue) (Merck Life

Science UK Limited, Gillingham) at a ratio of 1:1 sample/buffer. Then samples were boiled for 5 minutes and run in duplicate on two 12% Tru-page precast gels (Merck Life Science UK Limited, Gillingham) submerged in a in a running buffer with a pH value of 8.2 and a working strength of 1.2 M Triethanolamine, 0.8 M Tricine, 2.0% (w/v) SDS. Voltage was set to deliver a 150v current (20mAmp/gel). Electrophoresis was stopped as the leading-edge dye approached the bottom of the gel (about 2 cm). The first gel was submerged in Coomassie InstantBlue (Abcam, Cambridge) staining solution and incubated at room temperature, occasionally agitating until bands appeared (5-10 min) and stopped by gently rinsing in tap water. Subsequently, images were taken using a Biorad imaging system. Molecular sizes of proteins were estimated using a Novex Sharp pre-stained protein standard (Invitrogen) (Merck Life Sciencies UK Limited, Gillingham). The second gel was used for transferring proteins to a nitrocellulose membrane.

2.4 Western Blot and Immunoblot

Proteins separated under reducing conditions were transferred onto nitrocellulose a membrane using a Trans-Blot, Semi-dry Turbo Transfer System (Biorad, Watford, UK). The membrane was placed in a large white weigh boat (12 cm) and subsequently submerged in blocking buffer, covered, and incubated at 37 ^{O}C for 30 min. The membrane was washed three times in washing buffer for 3 min per wash. Subsequently, the fixed proteins were incubated with primary antibody (anti-lobster, raised in rabbit and extracted at 72 days) diluted in blocking buffer (0.3% PBS with Tween 20 and skimmed milk powder) at 1:400, then washed. The membrane was incubated in anti-rabbit secondary antibody diluted in 0.3% PBS Tween 20 at 1:400, washed again. The membrane was submerged in NBT/BCIP substrate buffer (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Merck

Life Science UK Limited, Gillingham) at a working strength of BCIP 0.15 mg/ml, NBT 0.30 mg/ml, Tris buffer 100 mM and MgCl2 5mM, pH 9.25–9.75, until proteins had developed (10-20 min). The reactions were stopped with deionised water and blot was viewed in a Biorad imaging system. This process is illustrated in the following diagram (figure 6).

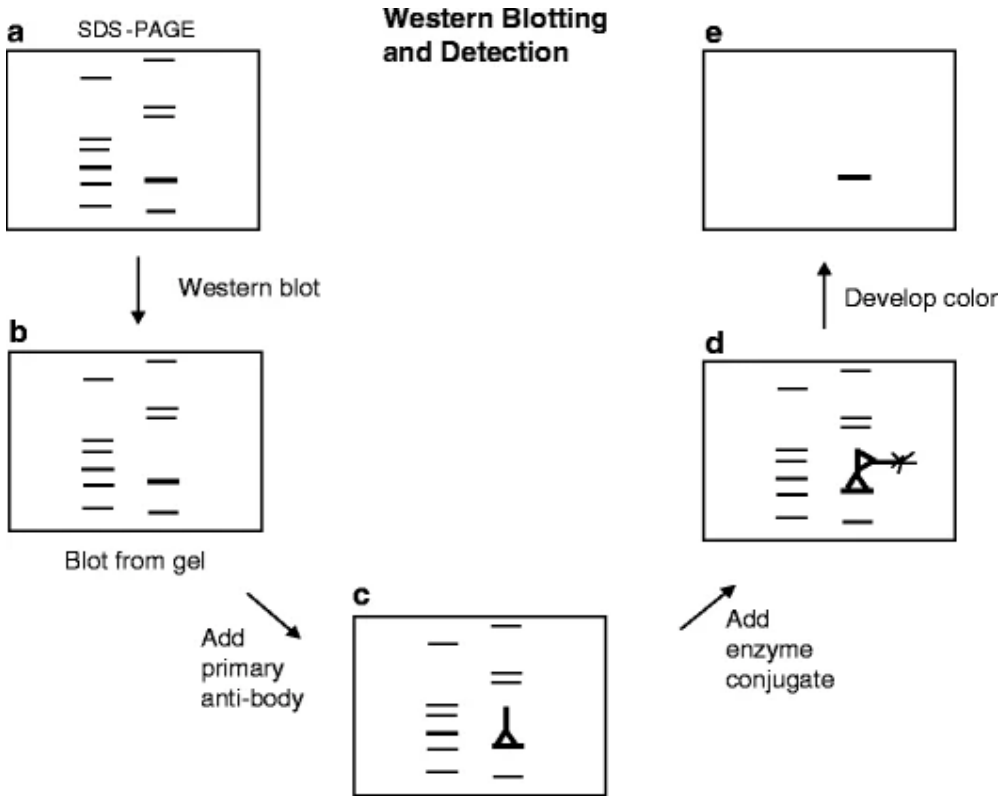


Figure 6. Diagram demonstrating steps in Western Blotting (Kurien *et al*., 2011)

2.5 Methodology for data analyses

All data analyses were performed using GraphPad Prism (Version 9.3.1). All data sets were primarily checked for normality using both the Shapiro-Wilk and Kolmogorov–Smirnov tests. Where data was not normally distributed, non-parametric tests were applied (Mann-Whitney, one way repeat measures ANOVA or unpaired nonparametric). Where data was normally distributed, parametric tests were used for analyses (one way ANOVA, paired t-

tests, interpolation and simple linear regression). Descriptive statistics were also used to reflect raw data in some cases. Error bars for non-parametric tests reflect the medians whereas parametric test error bars display the standard deviation.

3. Results

3.1 Optimisation

A Bradford assay estimated the concentration of crude lobster egg homogenate to be around 15mg/ml, and the subsequent supernatant of the same sample to be 10mg/ml based on interpolated data from a serial dilution of Bovine Serum Albumin (BSA) standard tested in the same assay. A trend in optical densities shows higher absorption with more concentrated samples, for example an optical density of 0.1380 was achieved with a standard concentration of 0.07800mg/ml (figure 7). The range in optical density for standards was between 0.1380 and 0.4415. For proteins, samples were tested neat, and diluted at 1:4 and 1:9 in dH_2O, giving optical densities of 2.1745, 0.7665 and 0.3995 respectively for crude antigen and 1.4240, 0.5015 and 0.2875 for supernatant samples.

With a concentration estimate known, a subsequent checkerboard titration to optimise the assay was carried out (figure 8a). The trend seen reflects the hypothesis in that the highest optical density (0.4660) is achieved with the most concentrated of the dilution series for both antigen and antibody (1:100) with a downwards trend observed as both antigen and antibody become diluted to 1:3200 (where OD is 0.1050). The control titration (where lobster egg homogenate was tested antigen was tested with normal rabbit primary antibody in place of anti-lobster egg antibody raised in rabbit) also showed higher optical densities with more concentrated antigen and antibody (highest was 0.2010 at 1:100 and 1:200 antigen and antibody respectively), however it is noted that there is less of a consistent trend (two optical densities above 0.1 where antigen dilutions are 1:800 and 1:3200) (figure 8a).

Based on this, an optimum dilution factor for both antigen (in BCB) and primary antibody (in 0.3% PBS Tween 20 with milk) would be selected and applied to all subsequent assays,

while dilutions for secondary antibody and substrate would remain fixed as described in the methods. The range between optical densities for the egg homogenate and control checkerboard was calculated for all dilution factors and hence the percentage of background determined. While higher signals were achieved from more concentrated dilutions, background signal was minimised to 14% at 1:400 for both antigen and antibody compared to 1:100, when compared to the same titration carried out with normal rabbit antibody as a negative control (figure 8b). Hence, it was found to be the most efficient dilution factor, whilst keeping antibody consumption to a minimum. This dilution factor was applied to all ELISAs throughout the study in both the development of controls and the testing of unknown samples.

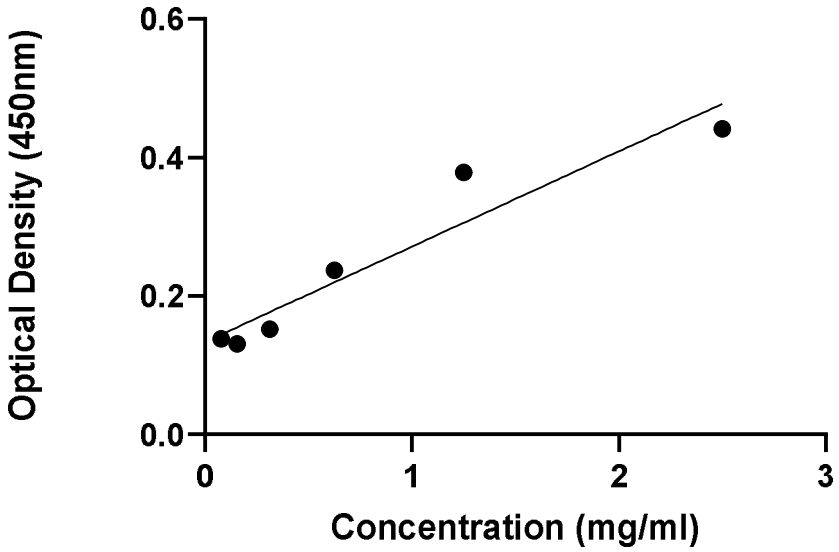
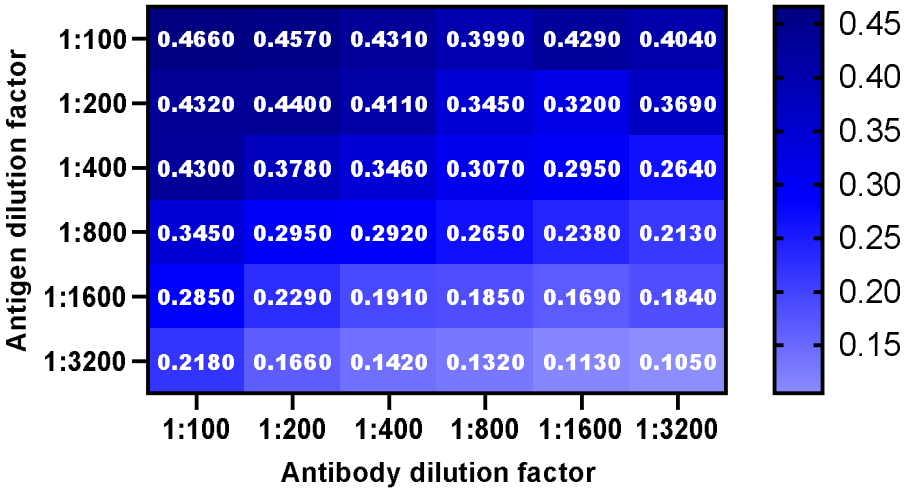


Figure 7. Concentration estimation plot from Bradford Assay carried out with lobster egg homogenate samples. Optical densities obtained from a set of standards show an upwards trend in a simple linear regression plot. Concentration estimates were extrapolated for unknown protein samples for both lobster egg homogenate and supernatant.



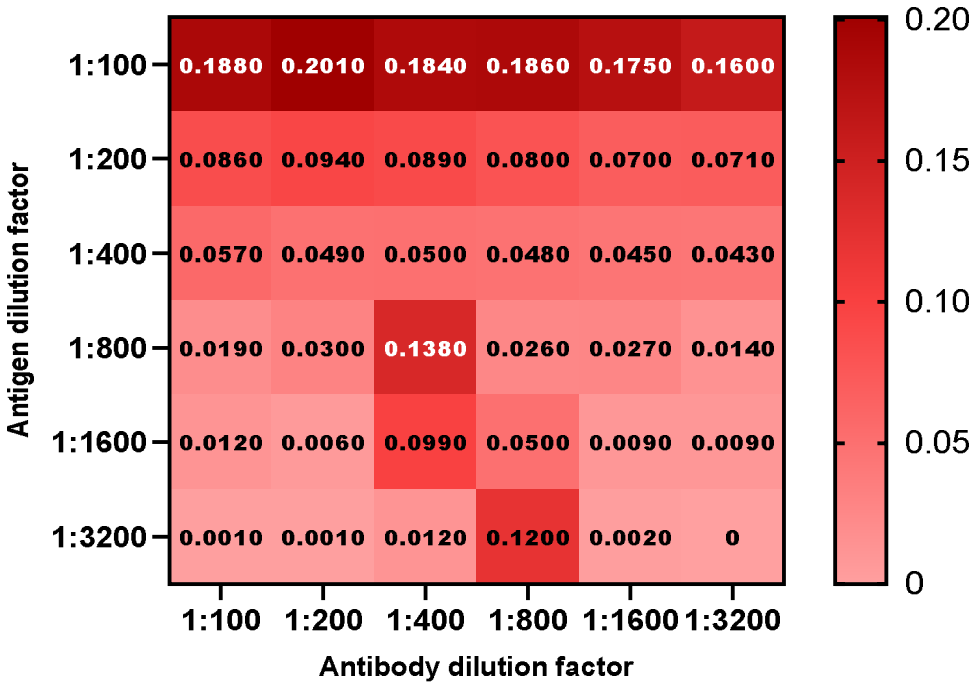


Figure 8a. & 8b.

8a. Heatmap illustrating optical densities from a titration where lobster egg homogenate antigen and anti-lobster egg homogenate primary antibody IgG are serially diluted. A marked decrease in optical density is seen as both antigen and antibody dilution factors increase.

8b. Heatmap illustrating optical densities from a titration where lobster egg homogenate antigen and normal rabbit primary IgG antibody are serially diluted. While some unexpected peaks are observed (for example, 1:800 Ag with 1:400 Ab), the general trend of this optical density background shows a decrease in OD as Ab and Ag dilution decreases.

3.2 Establishing Controls

Chosen dilution factors were applied to an optimised assay to demonstrate specific binding as well as a positive reaction between lobster egg homogenate (antigen) and the primary anti-lobster antibody. This test resulted in an average optical density of 0.2512 from 10 individual females sampled (figure 9) (these samples were also pooled and tested, giving an OD of 0.3630, this pooled antigen was used as a positive control throughout the rest of all assays. When replacing anti-lobster egg homogenate primary antibody with normal rabbit serum, optical density values are significantly different with an average OD of 0.0507 ($p=<0.0001$).

To establish a working negative control and cut-off value for testing unknown or suspected scrubbed female lobsters, an ELISA was carried out on swabs from 10 male lobsters. Optical densities were within the range of 0.06-0.0915, with a mean of 0.0709. From this, the negative cut-off value was calculated as 0.0989 using the mean value plus 3 times the standard deviation (this is a widely used method of determining negative cut-off values in ELISAs) (Lardeuz, Torrico & Aliaga, 2016). A higher level of background noise was noted with one male sample where the optical density was 0.0915 (figure 10). This calculated cut-off value provides a conservative estimate for determining whether a swab has picked up any lobster egg homogenate antigen in field testing.

Female lobsters swabbed immediately after having eggs scrubbed off gave a strong reaction for the presence of lobster egg homogenate protein (average absorbance from 6 specimens was 0.2325) (figure 11). A multiple comparison test shows no significant difference in ODs between 0-4 hours and 4-8 hours. However, there is a significant difference between 0 and 8 hours (*p=0.0189*). Hence, there is a clear downwards trend in

optical density over time which sees absorbance drop below the negative cut-off level 8 hours after scrubbing, but not necessarily after 4 hours. Despite this trend, one individual had an OD reading for the swab at 0 hours which was lower than the negative cut-off value (0.0785). The subsequent 4-hour swab then gave an OD of 0.2540 (above the cut-off value), dropping once more to negative after 8 hours (0.0610).

Overall results from male negative controls; normal rabbit primary antibody targeting lobster egg homogenate antigen, scrubbed females (mean OD of 3 swabs over 8 hours), and egg homogenate (targeted with anti-lobster egg homogenate raised in rabbits) were compared, which showed mean optical densities for both scrubbed females and lobster egg homogenate were higher than the negative cut-off value on average (figure 12). Although a strong signal is obtained from female swabs at both 0 and 4 hours (as described in figure 11), when an average is taken across 8 hours post-scrubbing, the mean optical density falls closer to the negative cut-off value. Both male swabs and normal rabbit serum were effective negative controls, the latter effective in representing specific binding of lobster egg homogenate to anti-lobster primary antibody, although the mean optical density was higher for swabbed males, suggesting more background noise.

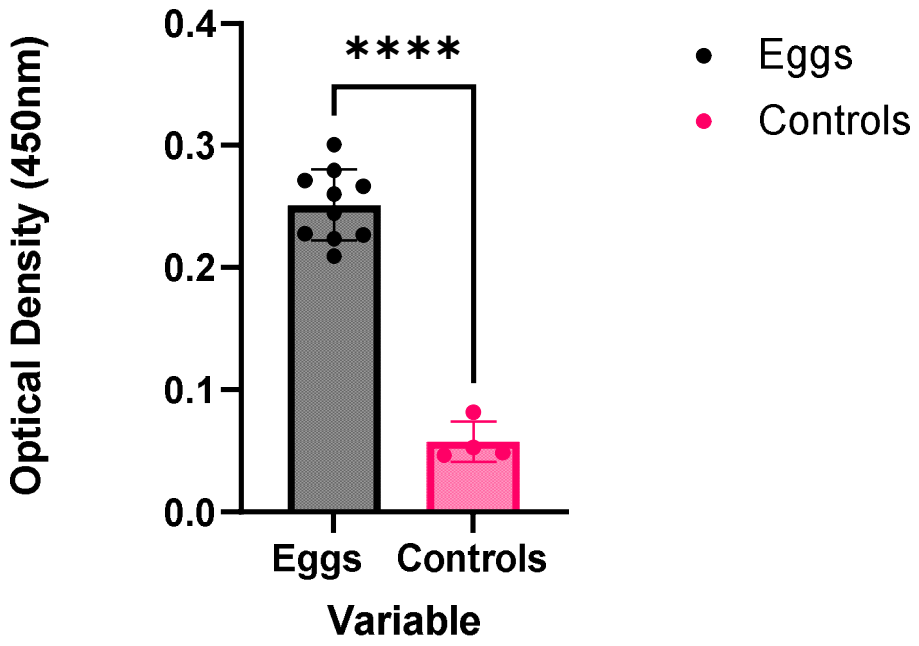


Figure 9. The overall difference in optical density from an ELISA where lobster egg homogenate antigen is targeted using anti-lobster egg homogenate primary antibody versus an ELISA where lobster egg homogenate is targeted using normal rabbit serum as primary antibody. There is an overall large and significant difference in optical density obtained from both variables. Significance was determined using a non-parametric unpaired t-test.

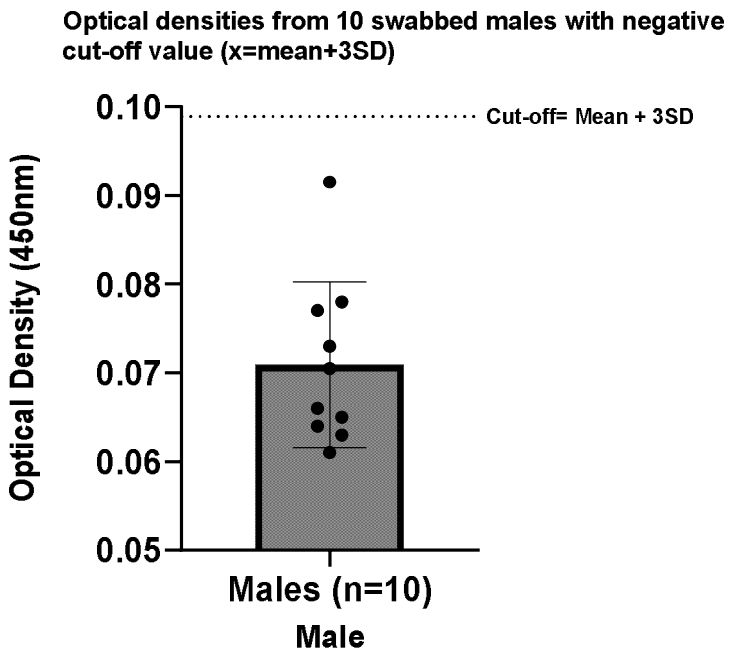


Figure 10. Optical densities from 10 swabbed male specimens as a negative control. These results show an average optical density for males to be 0.0709, with the highest value obtained as 0.0915- seen as a potential anomaly. A negative cut-off value has been calculated from these values which can be applied in further tests.

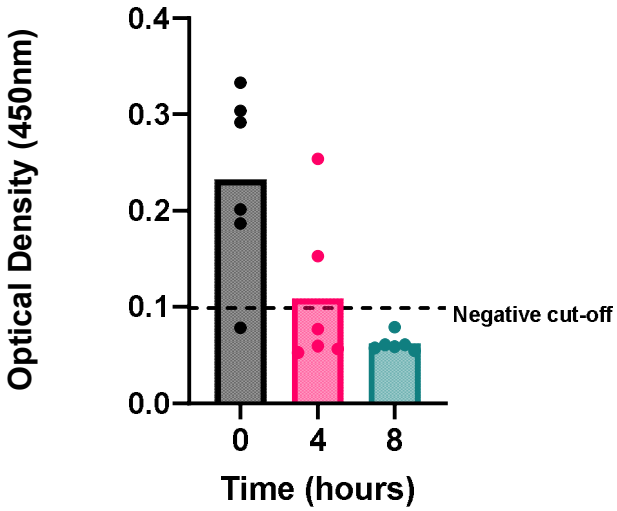


Figure 11. Optical density values for six scrubbed individuals over time with a calculated negative cut-off value line applied. This data shows a downwards trend in optical density over eight hours with a significant difference in optical density between the zero hours swab samples and the eight-hour samples. Significance was determined using a non-parametric repeated measuse one-way ANOVA.

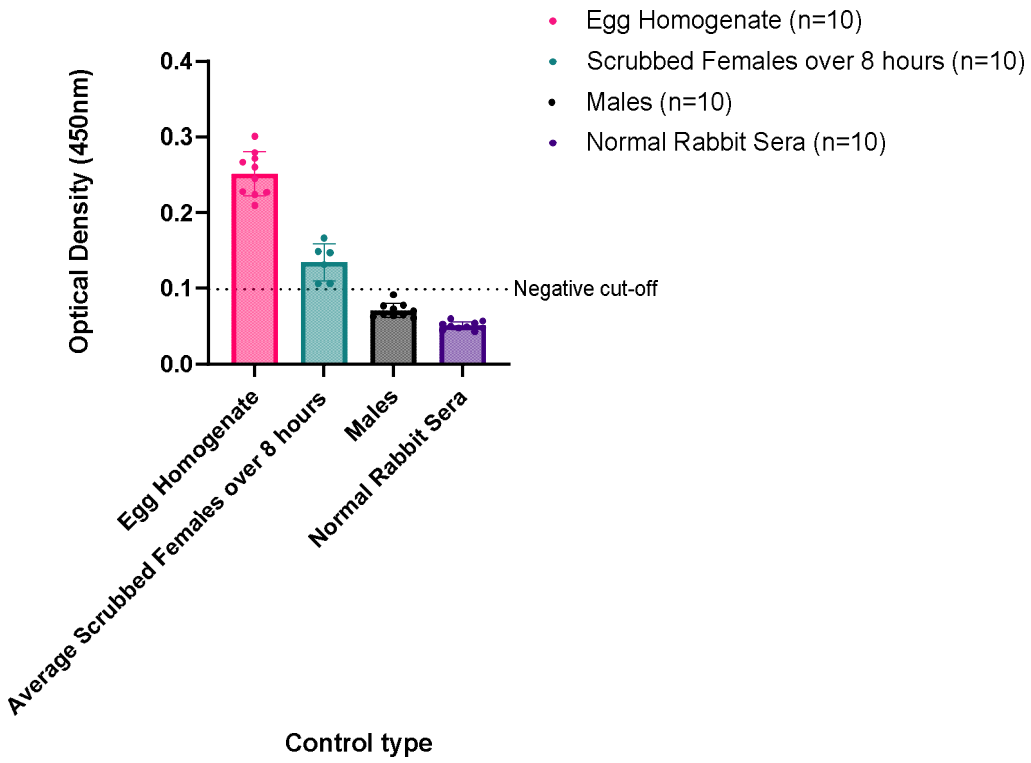


Figure 12. Comparisons in optical density from assays run on lobster egg homogenate, average values for scrubbed females over 8 hours, males and normal rabbit sera targeting lobster egg homogenate antigen. The negative cut-off value line is applied. Both the egg homogenate and scrubbed assays show average values above the negative cut off value. Male swabs compared to using normal rabbit sera show they function well as a negative control.

3.3 Analysis of different stages of embryonic development

Lobster egg homogenate samples for the same 10 individual hens samples previously from lobster egg homogenate were categorised according to the pregnancy stage of the individual based on information from sample providers at the NLH. Here, 'hatching' was characterised as the point at which eggs were harvested at a time where they would have been mature enough to hatch or have started hatching. The category of 'not hatching' was defined as a point where eggs were harvested and were not mature enough to hatch into juveniles. Analysis of optical densities from the ELISA assay where this was tested showed no significant difference in absorbance between individuals hatching and not hatching ($p=0.2032$), although overall optical density was lower for samples characterised as 'hatching' (figure 13).

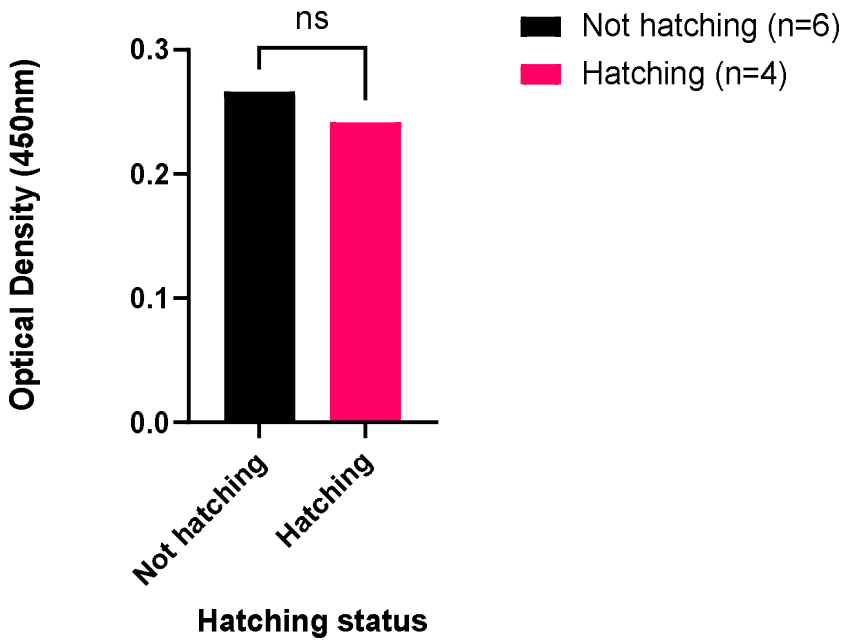


Figure 13. Mean difference in optical density between hatching and non-hatching lobster eggs when an ELISA is carried out targeting lobster egg homogenate antigen with primary anti-lobster

egg homogenate primary antibody. Significance was tested using a parametric paired t-test where was no significant difference in optical density between hatching and non-hatching eggs.3.4 Analysis of haemolymph samples

A comparison carried out between lobster egg homogenate and haemolymph samples from the same individuals (provided by the NLH) showed presence of the lobster egg homogenate proteins in the haemolymph of berried females (figure 14). Absorbance from all haemolymph samples fell above the negative cut-off value determined within the assay. Despite this, there is a significant difference in optical density mean between values for haemolymph and egg homogenate, giving a prevalent but still lesser ability to detect the protein in blood (p= <0.0001). Furthermore, there appears to be some correlation in signal between each individual, meaning a higher signal in egg homogenate samples is often met with a seemingly higher signal in haemolymph from the same individual.

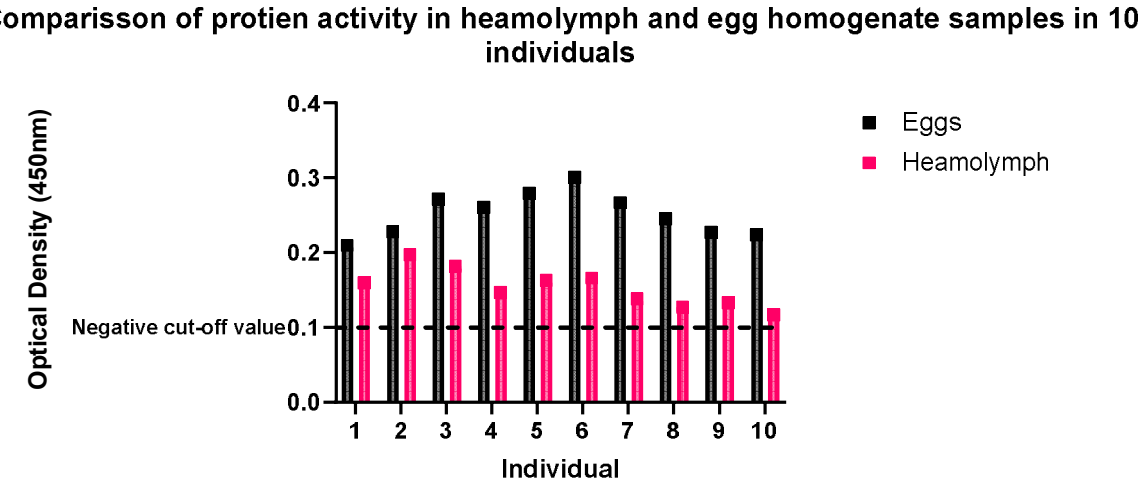


Figure 14. Both egg homogenate and haemolymph assay results for 10 berried individuals. The results show that while proteins can be detected in the haemolymph sample of berried females, the signal from egg homogenate is higher. Both haemolymph and egg values are above the negative cut-off value.

3.5 Field testing

Swabs from 50 female lobsters taken at a landing port were analysed in an assay to detect proteins from lobsters where it was unknown whether eggs had been scrubbed for each individual. A chart of all sample points shows one specimen to pick up a signal above the negative cut-off value of 0.098 (figure 15). Forty-nine of the swab samples fell below the negative cut-off value, with the majority of swabs achieving an optical density between 0.050-0.059. There is a consistent level of background noise from swabs deemed as 'negative' in these assays (figure 14). For each assay, a negative cut-off value was determined, and an average used for grouped analysis.

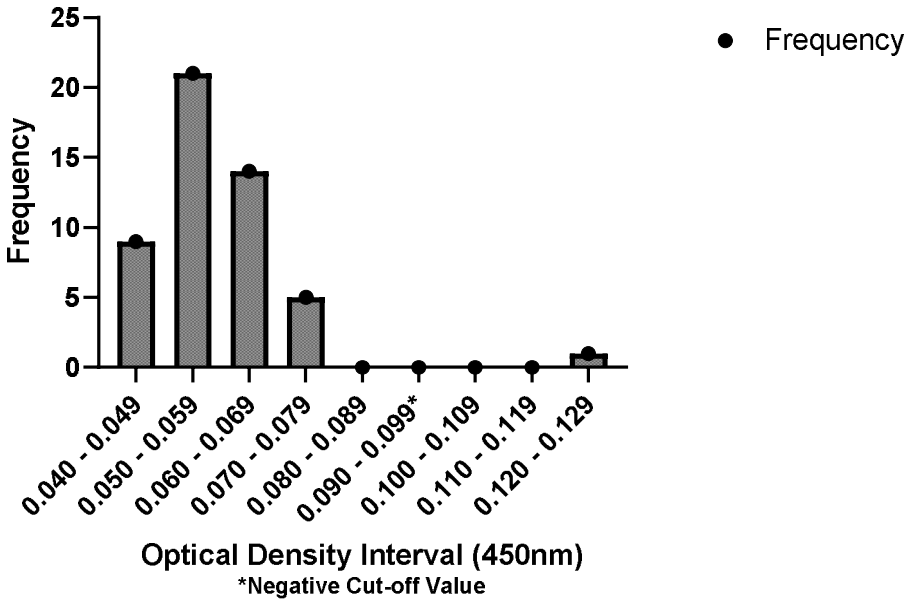


Figure 15. Frequency of optical densities from 50 swabs of females from a landing port where scrubbing status is unknown. The most frequent optical density value detected is 0.050-0.059. One sample was found to have an optical density above the negative cut-off value.

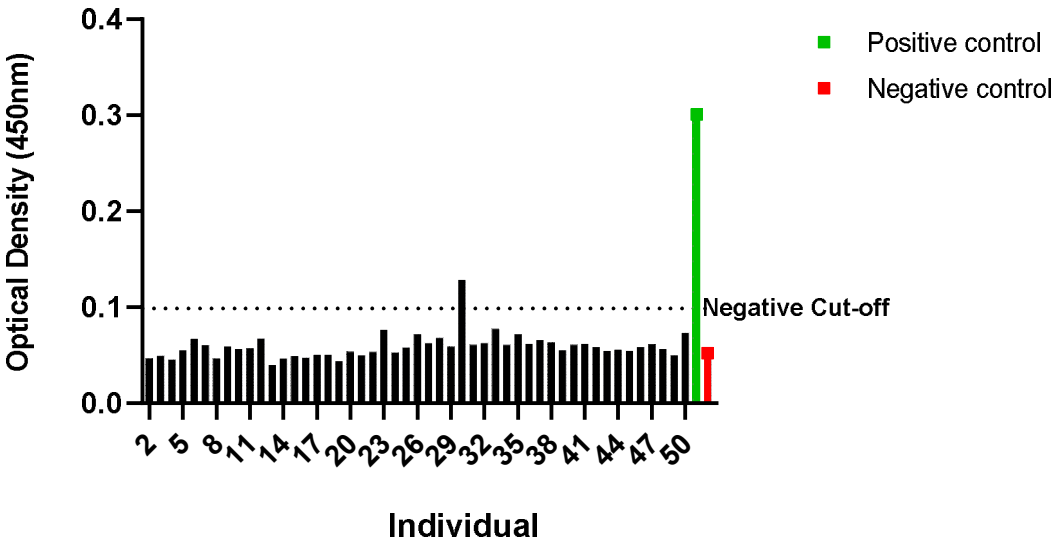


Figure 16. 50 swab samples of female lobsters where scrubbing status is unknown- represented by black bars. One specimen had an optical density over the negative cut-off value while all others remained consistently low.

3.6 Protein degradation

Swab samples taken of lobster egg homogenate antigen over 4 hours to determine whether protein degrades over time shows that in both dry (figure 17a) and wet (17b) conditions (technique described in the methods), there is a decline in optical density after 4 hours, suggesting protein degradation. In both assays, optical density readings are lower assays run on pure lobster egg homogenate samples (pure homogenate, pooled positive controls and scrubbed female lobster swabs give absorbance readings generally above 0.1). Nonetheless, optical density readings from the two different assays show protein activity declines more rapidly in wet conditions than dry where absorbance levels remain more

stable, with less readings falling below the negative control for the respective assay. In wet conditions, absorbance falls considerably lower than the normal rabbit sera control, suggesting there is no protein activity, after the 195-minute swab interval. The deviation from zero was determined to be 'not significant' in dry conditions (p= 0.0735) but significant in wet conditions (p=0.0001) further supporting this trend. Extrapolated y-values for dry conditions show a continuation of the downwards trend, predicting a decrease to 0.049 and below after 250 minutes.

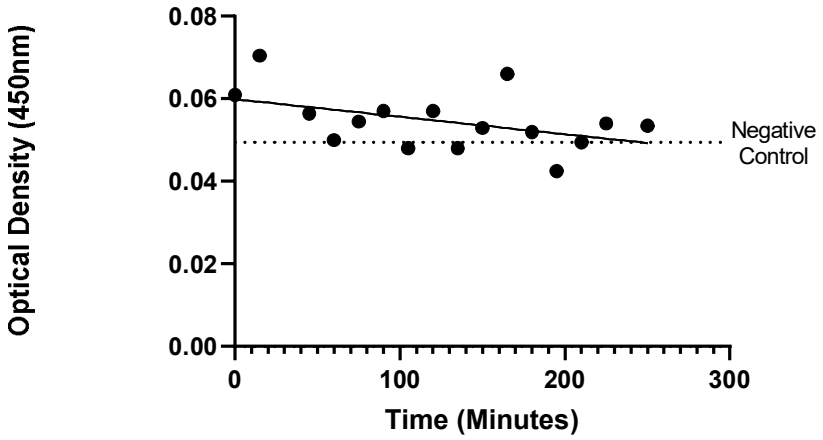


Figure 17a. Downwards decline in optical densities over 300 minutes from swabs taken from dry lobster egg homogenate antigen samples in in-vitro conditions. Deviation from zero is not significant using simple linear regression.

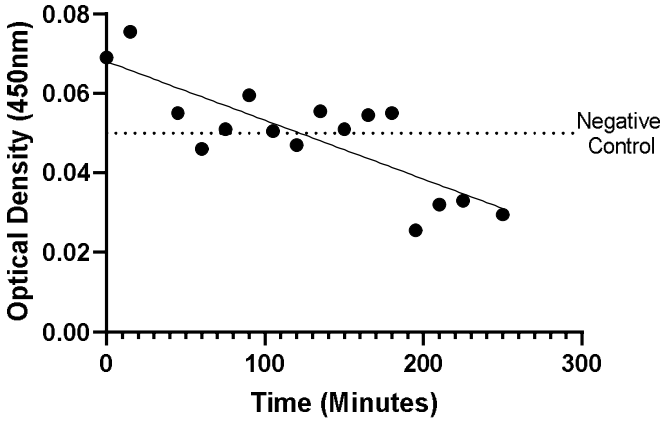


Figure 17b. Downwards trend in optical densities from swabs of lobster egg homogenate antigen sample over 300 minutes in wet, in-vitro conditions. Deviation from zero is significant using simple linear regression.

3.7 SDS-PAGE and Western Blot

Separated bands from SDS-PAGE and subsequent blotting show that for both crude antigen sample (egg homogenate) and supernatant (from the same sample), prominent protein bands appear at 40 kilodaltons (kDa), in accordance with the marker lane (M) (figure 18a). As the samples become more dilute across the lanes, as does the intensity of the protein bands. Results from immunoblotting show the same 40kDa bands to react when introduced to the lobster egg homogenate primary antibody and subsequent secondary conjugated antibody (figure 18b).

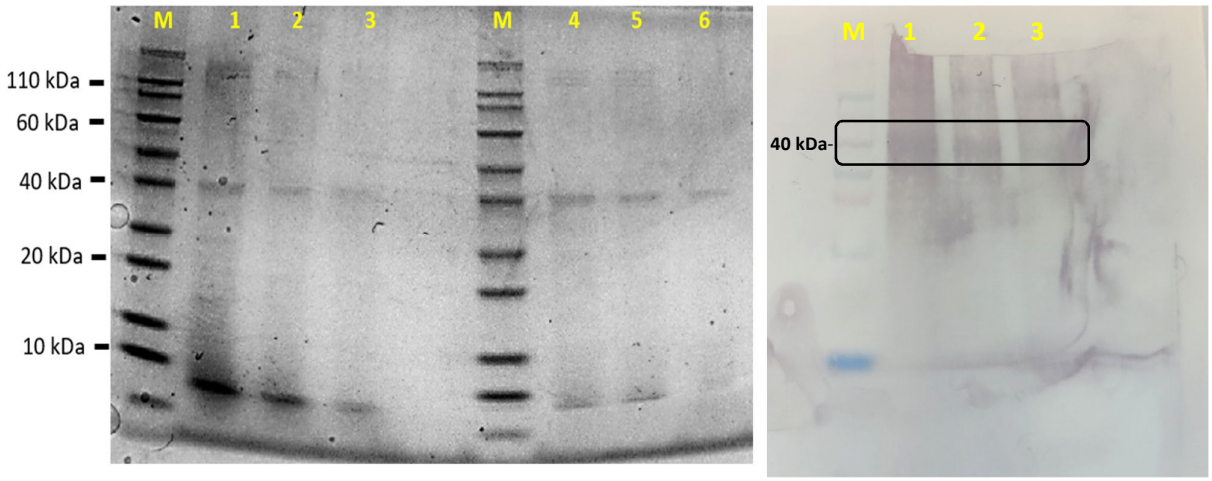


Figure 18a. Western Blot Images of crude antigen sample (left) and supernatant (right). For both samples, bands appear at 40 kDa and fade with concentration

Figure 18b. Immunoblot images showing reactions at the band size of 40 kDa

4. Discussion

The overall aim of this study was to develop a molecular tool to detect the illegal practice of scrubbing of berried lobsters landed in England. This was done for the first time, and the results represent a big step forward in the enforcement of the law of fisheries management, and hopefully to a more sustainable use of this iconic resource. The test was designed to detect the presence of trace egg proteins, left on the lobster carapace after the damage 'scrubbing' causes to the eggs. This study used the technique called ELISA, the building blocks used for quick assays like the human pregnancy test or a COVID antigen test, for its ease of application, quick delivery of results and because it would not require any special training for the enforcement officers. A protocol for the detection of egg proteins has been optimised through this study, on the surface of the carapace of a scrubbed lobster, assessing the sensitivity of this test and exploring the period this can reliably be applied for after scrubbing, given protein degradation.

4.1 The lobster pregnancy test

These results unmistakeably show that the illegal practice of scrubbing does indeed leave a protein trace on the surface of the lobster carapace, and that our ELISA protocol can detect the presence of this trace, easily, reliably, and quickly. This approach can provide results within 24 hours using the indirect ELISA as described here, but such protocol could easily be translated into more portable and accessible test, like a Sandwich ELISA, where the capture antibody is coated into the wells and analyte subsequently added during the assay. This would eliminate the need for overnight coating of antigen sample. Incubation times could also be reduced with further optimisation of the assay. In addition, further

study could introduce the use of the dot-blot assay whereby nitrocellulose can be used as a blotting membrane for antigen. This sample is then submerged in the same primary and secondary conjugated antibody as in the indirect ELISA, with shorter incubation periods (this can be carried out at ambient temperatures). A colour change on the nitrocellulose upon the introduction of substrate would indicate the presence of lobster egg proteins. This technique can be implemented in field conditions with minimal equipment and facilities. Indeed, this molecular tool has many applications, including a possible eventual transfer into a rapid lateral flow test which would prove to be the most beneficial in terms of cost and time when being used in the field.

4.2 Signal strength, false positives and false negatives

The 'signal to noise ratio' can be determined from the level of absorbance (optical density value) in negative controls compared to that of the samples. Indeed, the use of negative controls in each assay is imperative to avoid false positives by determining the assay is functioning properly. This determination can be used to support a low false positive rate when using this particular assay. However, this study shows that the probability of false negatives increases as time since scrubbing increases. Signal strength is at its highest the lesser the time between scrubbing and swabbing. This is shown from the decrease of optical density in scrubbed female lobster swabs over time where absorbance drops to levels below the pre-determined negative cut-off value (relative to the assay). Here, scrubbed females should yield positive results but appear to suggest no scrubbing has occurred.

ELISAs carried out with normal rabbit antibody (the rabbit has not been allowed to produce any polyclonal lobster egg homogenate antibodies) to replace anti-lobster egg

homogenate antibodies show only background levels of absorbance. Therefore, this study provides evidence that specific biding occurs between antigen in lobster egg homogenate and the anti-lobster antibodies used, as only background signal is detected when the plate is processed. This lack of binding indicates that using normal rabbit primary antibody is a credible and usable control to confirm an assay is working.

4.3 Protein Degradation

The results obtained in this study show that the positive signal produced by a scrubbed lobster are stronger soon after scrubbing, when the protein traces are fresh. Then, like many biological traces, proteins will start decaying. An investigation has started into this process, in order to understand how long after scrubbing the positive signal persists for. The time passing between the scrubbing and the swabbing is indeed an important factor: lobsters are often kept in pots, tanks or boxes, in wet or dry conditions, for a time after being caught and/or scrubbed, especially in the case of small operation fisheries. This could increase the risk of false negatives as a result of protein degradation. Degradation of proteins overtime would mean the shape of the antigen denaturing due to the lack of nutrient replenishment or change in conditions and temperature, making the probability of primary anti-lobster egg antibody binding the antigen sample much less likely, even though the protein may be present as a result of scrubbing. The downwards pattern in optical density over time from scrubbed females is likely attributed to this protein degradation. This is potentially in combination with the fact that scrubbed females were stored in tanks between swabs, which may have led to traceable proteins being manually removed by the water. The effect storage conditions have on this protein has also therefore been brought into question.

Thankfully, while optical density does decrease over time in tests, optical density remains above the noise level determined by the negative samples included, showing that if positive, a sample can be identified as such for up to four hours after scrubbing. With time after scrubbing, another factor that might affect the efficacy of this test is the mode of storage. As mentioned above, live lobster can be stored for some time in boxes or tanks before being sold, and potentially after scrubbing. Wet and dry conditions where roughly simulated in the lab using egg extract swabbed over glass slides that where then stored on the lab bench at room temperature or in water. They were then swabbed again, and the signal was checked. While the proteins still gave a positive hit after 4 hours in dry conditions, this time was reduced by about half in wet conditions. This could be due to both degradation and dilution of course. Some future work should be done to optimise this further and ensure that the amount of dilution during in-vitro experimentation is playing no role in the trends seen here.

Variations in storage conditions may affect residual proteins on the abdomen of the lobster could also determine the state of the proteins at the time of swabbing. Lobsters stored in tanks of seawater may have a greater probability of preserving the proteins as this is a closer condition to the natural state of the eggs. However, there is also the possibility this may wash off enough proteins to make them undetectable. Conversely, dry conditions may cause degradation due to potentially higher temperatures and difficulty in swabbing non-wet sample. Study on how temperature affects the state of proteins is also required to determine variations in degradation time as atmospheric temperatures vary with climate and seasonal changes. This was of course a first, rough attempt, but gives important information on what could be expected in field conditions.

Another possible explanation for this decreasing trend is the decreasing amount of proteins available to swabs over time. Swabs were carefully taken between every segment of the abdomen to maximise the probability of picking up antigen sample, rather than just running the swab lightly up and down in a vertical motion. This comprehensive sampling technique may have meant the maximum amount of potential antigen sample could have been collected during the first swab, leaving less and less for the subsequent time replicates. However, swabbing multiple specimens over time periods, or using certain sections of the abdomen each time may have meant unrepresentative sampling due to variations in the distribution of antigen amongst individuals. While taking multiple swabs of the same individual at the same time intervals may produce more representative sampling, there is also a suggestion that the same 'removal of proteins' could arise from repeated swabbing, potentially giving false negative results. If multiple swabs will be necessary in the field, a methodical subdivision of the surface of the carapace in zones could alleviate this problem.

Further, a potential discrepancy in swabbing technique and the amount of antigen sample picked up between swab intervals, may give rationale behind the increase in optical density that occurred in one specimen from 0 hours to 4 hours (figure 11), and hence the sudden drop back to negative absorbance values after 8 hours. For ethical reasons, there was a limit to the number of berried females that could be scrubbed. Nonetheless, a greater sample size would increase evidence of this potential occurrence.

After false negatives, false positives might represent a problem and it is of course important to explore the chances of incurring into them. One of the first sources of false positive explored is that of 'contamination' between a berried lobster and a non-berried

one, while stored in the same container. While further research is needed, it is unlikely that lobsters placed in the same holding environment contaminate one another, especially in the abdominal area as eggs are separated and well protected by the hen. However, given the potential applicability of this test in fisheries enforcement, it important to understand the extent of this phenomenon. Experimental investigation should be included (easily storing a berried female and a male in the same tank and taking routine swabs) to understand if this is a problem worth considering.

Lastly, it is important to rule out the possibility of detecting a false positive after natural hatching. Also, if swabs from recently spent lobsters provides an optical density reading above the threshold of the determined negative cut-off value, it is key to deduce the length of time the protein is detectable after the eggs have been hatched, as this may inflate the false positive rate. This was outside the scope of this study but discussed at length with the interested parties. The National Lobster Hatchery will play a crucial role in this as they do have berried lobster in their tanks which are kept for a few days after hatching.

4.4 Protein detection across embryonic development

Samples provided by the National Lobster Hatchery were analysed not only for detection of antigen, but the information provided about the eggs were implemented into answering questions about optical density at different stages of embryonic development. Given that eggs categorised as 'hatching' did not have significantly different optical densities from ELISAs using their crude antigen sample to eggs which were categorised as 'not-hatching', a preliminary determination can be made that the stage of embryonic development has no impact on the ability to detect a scrubbed lobster. These results may further indicate that ELISAs carried out at this concentration are functioning at the maximum detection rate,

whereby the highest possible rate of binding is taking place in the wells regardless of whether the amount of protein activity as a result of embryonic development stages is different.

4.5 The role of Haemolymph to detect lobster pregnancy

Haemolymph samples collected from berried females show the same proteins from lobster egg homogenate assays can be detected using the same anti-lobster egg primary antibodies used in crushed egg assays. This is due to the need of continuously replenishing nutrients for the embryo to develop. Even though this adds operational complication to the work of an enforcement officer, it could be used as a tool to validate and confirm a positive result when needed. Again, if used in law enforcement, it will be important to understand if the signal persists in the blood after naturally hatching and if yes, for how long.

4.6 Future protein characterisation

SDS-PAGE and western blotting shows protein bands can be separated in a blot and targeted in an immunoblot with anti-lobster egg homogenate antibody to identify reactive bands of prominent proteins. This may aid characterisation of the proteins within lobster egg homogenate in future research. These bands account for any proteins found in crushed lobster eggs including the outer case and cement substance which aids in holding eggs together.

4.7 Building upon current literature

This work builds upon existing literature about the detection of illegally landed lobsters. Detection of illegally harvested lobsters using a swabbing technique has been explored before. One study examines the efficacy of the starch-iodine swab technique to detect the use of bleach in catching spiny lobsters throughout the Bahamian Archipelago. Here, chemicals such as bleach, dishwashing liquid and gasoline are injected into a lobster den, triggering an escape response, and subduing the lobster- making them easier to catch (Baker 2008). This test was also applied to detecting the chemical removal of eggs from ovigerous lobsters, again using a starch-iodine swab test to determine the presence of bleach (Heckman et al., 2000). Prior to this, microscopy had been used to examine the pleopod for damage to the plumose setal hairs (Cogger and Bayer, 1996). In order to avoid the removal of a pleopod (invasive and can affect the value of the catch), a dye test was developed whereby 0.1% haematoxylin was applied to the abdomen of the lobster with a purple colour change from the tissue indicating the presence of bleach (Smith, 1999).

This study builds further upon existing and standard tests for the detection of female lobsters who have had eggs forcibly removed. The Karlson-Sissson (1970) test and work from the mid-1900's, look at the physical indicators of scrubbing using both haematoxylin and microscopy to examine damage to the pleopods caused by scrubbing (Nicosia & Lavalli, 1999). While this provides a strong foundation for detecting scrubbing, this study may allow for the further fortification of the argument and subsequent prosecution of these practices.

Although there is no previous literature to show an ELISA being used to target the proteins within lobster eggs, ELISAs have been used to detect other proteins common in

crustaceans. For example, an ELISA was used to detect tropomyosin in crustaceans such as shrimp, crab and lobster in processed food for allergens. This successfully measured the presence of the crustacean protein in foods where it had been declared and an absence where it had not been declared (Seiki *et al.,* 2007). There were no false positives for this study, fortifying the reliability of it when looking for the presence or absence of specific proteins.

Other research has investigated the changes in concentrations of digestive enzyme activity within embryos during development and newly hatched larvae of the tropical spiny lobster (*Panulirus ornatus*) using fluorescence-based assay techniques. Here, microscopy was used to determine the stage of embryonic development of lobster eggs and subsequent homogenization of egg samples were measured for both enzyme activity and protein content. The study noted a significant difference in ontogenic variation in enzyme activity with a general increase in digestive enzyme activity with embryonic development (Genodepa *et al.,* 2022). This similar use of technique and more in-depth look at embryonic development provides a key foundation for further study on the effects of embryonic development on the ELISA test in this study. However, detection using the ELISA technique may occur regardless of protein content due to the sensitivity of the assay.

This study builds upon literature, for example, Sibert, Ouellet & Brêthes (2004) find in their research a rapid decline in total yolk proteins during embryonic development. While this study suggests there is a decrease in protein detection- shown in the hatching and non-hatching- there was also found to be no overall significant difference in optical density between the two states. Further study is needed as this study does not show a comprehensive image of all stages of lobster embryonic development. However, it is

possible that the ELISA is at a level of sensitivity which detects protein to a point of saturation, so it may not be the case that optical density is proportionate to the amount of egg protein in sample but is able to detect it to give a positive result.

4.8 Future work

This study provides the first protocol that has been optimised to detect scrubbing on landed lobsters. Results unmistakeably show that this approach will detect traces of egg proteins left after scrubbing on the carapace of the lobster, for up to a maximum of four hours. Above we have discussed at length all the factors that might affect these results, with both the possibility of delivering false positive or negative errors. Many of these factors (like protein degradation) have been explored within this study, but much more should be done for this tool to become instrumental in law enforcement.

While providing solid background for the deployment of a reliable tool to detect illegal scrubbing, further work should be carried out to strengthen this tool. The main weakness is protein degradation, as a factor of time since scrubbing. While we can reliably detect eggs proteins soon after scrubbing, the signal tends to weaken and then disappear after two hours in wet conditions (spanning the timescale that lobsters would be scrubbed and then stored in a water tank until entering the supply chain) and four in dry conditions. This was based on a limited sample size and experimental design and future effort should be devoted to clarifying the fate of egg proteins under different conditions. In order to do that, identifying the protein (or proteins) involved in the signal detected here would be crucial. Given the lack of knowledge about the proteins contained in lobster eggs, our protocol used an egg homogenate to produce antibodies that are in turn used in the ELISA.

This means that proteins detected using this ELISA are not currently characterised but represent the entirety of the egg homogenate.

A specific monoclonal antibody or the current polyclonal anti-lobster egg homogenate antibody have the potential to be applied to a nitrocellulose membrane and used in a lateral flow test, which uses the same basic principle as an ELISA to target a specific analyte, giving a qualitative result which can be or quantitative when combined with reader technology. This rapid, cost-effective test development would require limited training to carry out and provide an initial indication of the presence of lobster egg protein, mandating for further collection of evidence.

In the development of a kit to test for scrubbed lobsters, it is conceivable that the argument may be made to suggest contamination of one female lobster with another. This could be in the form of a spent lobster in the same pot as a male or non-pregnant female, or a berried female landed in the same catch as male or non-pregnant females where eggs may have broken or rubbed against other individuals. There are several suggestions for future in-vitro research experiments including holding a series of combinations of individuals in the same tank. For example, a berried female with a male, a scrubbed female with a male and a recently spent female with a male. There should also be a non-pregnant female replacing the male in these combinations, to eliminate any behavioural discrepancies. The abdomens of male or non-pregnant females should be swabbed, and assays run to detect egg proteins. Furthermore, the outside of eggs could be swabbed to determine whether non-broken eggs in contact with another surface could create any signal detection. An absorbance below the negative cut-off value in this would eliminate the potential for the detection of the protein on individuals that have made contact with non-pregnant females.

In-vivo tests may involve swabbing specimens on fishing vessels. For example, when a pot is landed with a berried female, all specimens in the pot would be swabbed to determine whether the berried hen has caused any spread of lobster egg homogenate proteins.

While this study has been able to confirm the presence of lobster egg homogenate protein in haemolymph samples using the ELISA, more work must be done to ensure this protein cannot be detected post-hatch, leading to false positives. A series of samples taken over the course of the pregnancy of several female lobsters, including samples before fertilisation should be tested to create a timescale of protein detection levels over this period. Furthermore, detection of the pregnancy proteins in haemolymph of berried or suspected berried females could provide important evidence of pregnancy given the rate of protein degradation after 8 hours, subject to further testing on the levels of protein before and after the pregnancy.

4.9 The role of hatcheries

One of the main challenges throughout this study is the limitation in sample size. For testing scrubbed females, a total of six samples were available. This limit is partially due to the overall size of catch from the hatchery at the time of sampling and the need to retain several berried females for hatching and rearing to support stocks; as well as the ethical considerations taken in removing eggs from females. The specific limitation of this is in interpreting results of optical density over time. A more comprehensive data set is required to explicate the apparent fall in density over time, however the method of data collection is still debatable. In this regard, lobster hatcheries will play a vital role, in addition to that of support to replenishing stocks. Their knowledge and exclusive access to samples under different conditions will be very important in filling the gaps thar remain after this project.

4.10 A simple solution for an international problem

This project was started to support enforcement officers working in English landing ports. Nevertheless, scrubbing is known to be carried out in other parts of world. Reports of eggs removal indicate that this practice happens in the United States of America too. Here, eggs are not removed by mechanical means, but the tails dipped in a bleach and water solution causing a removal of eggs from the abdomen within 2 minutes (Cogger & Bayerz, 1996).

Further applications towards developing a body of evidence to enforce 'The Lobsters and Crawfish (prohibition of landing) (amendment) Order 2017' may involve the swabbing of tools on the fishing vessel which could have encountered the berried hen during the scrubbing process. This may include high pressure hoses or the bristles of the scrubbing brush. Swabs would be taken using the same protocol as swabbing for berried females, and run using the same ELISA protocol, targeted with anti-lobster egg homogenate primary antibody.

4.11 Concluding remarks

It is hoped that this study may contribute to the future implementation of a rapid molecular test, commonplace in the detection of scrubbed female lobsters. This test would not only the allow for the enforcement of this law but also act as a potential deterrent to the breaking of it. Overall, this would reduce the exploitation of this important and valuable resource and even potentially inspire future projects to apply molecular techniques to supporting fisheries both in the UK and around the world.

References

Acheson, J., & Gardner, R. (2011). The evolution of the Maine lobster V-notch practice: cooperation in a prisoner's dilemma game. Ecology and Society, 16(1).

Attard, J., & Hudon, C. (1987). Embryonic development and energetic investment in egg production in relation to size of female lobster (*Homarus americanus*). Canadian Journal of Fisheries and Aquatic Sciences, 44(6), 1157-1164.

Badger, B., Consultation on the prohibition on landing egg bearing lobsters and crawfish in England (2017). DEFRA. Retrieved from https://www.legislation.gov.uk/uksi/2017/899/pdfs/uksiod_20170899_en.pdf

Baker, C. S. (2008). A truer measure of the market: the molecular ecology of fisheries and wildlife trade. *Molecular Ecology*, *17*(18), 3985-3998.

Bannister, R. C. A., Addison, J. T., & Lovewell, S. R. J. (1994). Growth, movement, recapture rate and survival of hatchery-reared lobsters (Homarus gammarus (Linnaeus, 1758)) released into the wild on the English east coast. Crustaceana, 156-172.

Cefas, 2020. Lobster (Homarus gammarus) Cefas Stock Status Report 2019 October 2020. [online] Available at:<https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/722748/2017_Lobster_assessments.pdf> [Accessed 26 August 2022].

Carere, C., Nascetti, G., Carlini, A., Santucci, D., & Alleva, E. (2015). Actions for restocking of the European lobster (Homarus gammarus): a case study on the relevance of behaviour and welfare assessment of cultured juveniles. Rendiconti Lincei, 26(1), 59-64.

Cogger, E. A., & Bayer, R. C. (1996). Detection of egg removal from the ovigerous lobster following chlorine bleach exposure. *J. Interdiscip. Stud*, *9*, 65-70.

Crimmins, E. (2018). The Influence of the Lamlash Bay no-take zone, Firth of Clyde, on spatial and Temporal Variation in the Recovery of Commercially Exploited Crustaceans (Doctoral dissertation, MSc Thesis North Yorkshire: University of York, 43).

Crowther, J. R. (2008). *ELISA: theory and practice* (Vol. 42). Springer Science & Business Media.

DeAngelis, B. M., Cooper, R., Clancy, M., Cooper, C., Angell, T., Olszewski, S., ... & Catena, J. (2010). Impacts of v-notching the American lobster. *Journal of Shellfish Research*, *29*(2), 489-496.

De Grave, Pentcheff, N. D., Ahyong, S. T., Chan, T.-Y., Crandall, K. A., Dworschak, P. C., Felder, D. L., Feldmann, R. M., Fransen, C. H. J. M., Goulding, L. Y. D., Lemaitre, R., Low, M. E. Y., Martin, J. W., Ng, P. K. L., Schweitzer, C. E., Tan, S. H., Tshudy, D., & Wetzer, R. (2009). A Classification of Living and Fossil Genera of Decapod Crustaceans. The Raffles Bulletin of Zoology.

Dehn, P. F., Aiken, D. E., & Waddy, S. L. (1983). Aspects of vitellogenesis in the lobster Homarus americanus. Fisheries and Environmental Sciences, Department of Fisheries and Oceans, Biological Station.

Direct and Indirect ELISA. (2022). Thermofisher Scientific. Retrieved 6 September 2022, from https://www.thermofisher.com/nl/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

Ellis, C. D., Hodgson, D. J., Daniels, C. L., Boothroyd, D. P., Bannister, R. C. A., & Griffiths, A. G. (2015). European lobster stocking requires comprehensive impact assessment to determine fishery benefits. ICES Journal of Marine Science, 72(suppl_1), i35-i48.

Emre, Ö. Z. E. R., ACARLI, S., & Berber, S. (2020). Preliminary results on the growth of larval European lobster (Homarus gammarus (Linneaus, 1758)) in Turkey. Marine Science and Technology Bulletin, 9(1), 62-70.

Erkan, M., & Ayun, Y. (2014). Morphological and histochemical examination of male and female gonads in Homarus gammarus (L. 1758). Open Life Sciences, 9(1), 37-48.

Factor, J. R. (Ed.). (1995). Biology of the Lobster: Homarus americanus. Academic Press.

Genodepa, J., Zeng, C., Militz, T. A., & Southgate, P. C. (2022). Ontogenetic variation in digestive enzyme activities within embryos and newly-hatched larvae of the tropical spiny lobster, Panulirus ornatus. *Aquaculture*, *548*, 737595.

Gerlach, G., & Artema, J. (2012). The use of chemical cues in habitat recognition and settlement. Chemical ecology in aquatic systems, 72-89.

Goetting, K., & Whiffen, C. (2011). The effects of v-notching on reproductive potential in American lobsters (Homarus americanus) in Newfoundland.

Hinchcliffe, J., Agnalt, A. L., Daniels, C. L., Drengstig, A. R., Lund, I., McMinn, J., & Powell, A. (2022). European lobster *Homarus gammarus* aquaculture: Technical developments, opportunities and requirements. Reviews in Aquaculture, 14(2), 919-937.

Heckman, K. W., Smith, S. L., Bayer, R. C., Jahngen, E. G., & Smith, F. X. (2000). Starch–Iodide Swab Technique for Detecting Bleach-Dipped Female Lobsters. North American Journal of Fisheries Management, 20(2), 538-541.

House of Commons Library (2022) *'UK fisheries statistics'*. London: House of Commons Library. Available at: https://researchbriefings.files.parliament.uk/documents/SN02788/SN02788.pdf [Accessed 17 November 2022]

Jenkins, T. L., Ellis, C. D., Durieux, E. D., Filippi, J. J., Bracconi, J., & Stevens, J. R. (2020). Historical translocations and stocking alter the genetic structure of a Mediterranean lobster fishery. Ecology and evolution, 10(12), 5631-5636.

Johnson, K. J., Goldstein, J. S., & Watson III, W. H. (2011). Two methods for determining the fertility status of early-stage American lobster, Homarus americanus, eggs. Journal of Crustacean Biology, 31(4), 693-700.

Jørstad, K. E., Prodohl, P. A., Agnalt, A. L., Hughes, M., Farestveit, E., & Ferguson, A. F. (2007). Comparison of genetic and morphological methods to detect the presence of American lobsters, Homarus americanus H. Milne Edwards, 1837 (Astacidea: Nephropidae) in Norwegian waters. Hydrobiologia, 590(1), 103-114.

Karim. (2018). ELISA. Nova Science Publishers, Incorporated.

Karlsson, J., & Sisson, R. (1973). A technique for detection of brushed lobsters by staining of cement on swimmerets. *Transactions of the American Fisheries Society*, *102*(4), 847-848.

Kleiven, A. R., Espeland, S. H., Stiansen, S., Ono, K., Zimmermann, F., & Olsen, E. M. (2022). Technological creep masks continued decline in a lobster (Homarus gammarus) fishery over a century. Scientific reports, 12(1), 1-10.

Kurien, B.T., Dorri, Y., Dillon, S., Dsouza, A. and Scofield, R.H., 2011. An overview of Western blotting for determining antibody specificities for immunohistochemistry. *Signal Transduction Immunohistochemistry*, pp.55-67.

Lardeux, F., Torrico, G., & Aliaga, C. (2016). Calculation of the ELISA's cut-off based on the change-point analysis method for detection of Trypanosoma cruzi infection in Bolivian dogs in the absence of controls. Memórias do Instituto Oswaldo Cruz, 111, 501-504.

Laverdure, A. M., & Soyez, D. (1988). Vitellogenin receptors from lobster oocyte membrane: solubilization and characterization by a solid phase binding assay. International journal of invertebrate reproduction and development, 13(3), 251-266.

Lin, A. V. (2015). Indirect Elisa. In *ELISA* (pp. 51-59). Humana Press, New York, NY.

Lizarraga-Cubedo, H. A., Tuck, I., Bailey, N., Pierce, G. J., & Kinnear, J. A. M. (2003). Comparisons of size at maturity and fecundity of two Scottish populations of the European lobster, *Homarus gammarus*. Fisheries Research, 65(1-3), 137-152.

Marine Management Organisation. (2018, June 8). R130: *Detecting berried lobsters.* https://www.gov.uk/government/publications/r130-detecting-berried-lobsters/r130-detecting-berried-lobsters

Mellinger, J. (1995). Les réserves lipidiques de l'œuf des poissons. Année Biologique, 34(2), 63-90.

Moland, E., Ulmestrand, M., Olsen, E. M., & Stenseth, N. C. (2013). Long-term decrease in sex-specific natural mortality of European lobster within a marine protected area. Marine Ecology Progress Series, 491, 153-164.

Moland, E., Fernández-Chacón, A., Sørdalen, T. K., Villegas-Ríos, D., Thorbjørnsen, S. H., Halvorsen, K. T., ... & Knutsen, J. A. (2021). Restoration of abundance and dynamics of coastal fish and lobster within northern marine protected areas across two decades. Frontiers in Marine Science, 8, 674756.

Nicosia, F., & Lavalli, K. (1999). Homarid lobster hatcheries: their history and role in research, management, and aquaculture.

NIFCA, 2017. NIFCA V-notching 2017 Report. [online] NIFCA. Available at: <https://nifca.gov.uk/wp-content/uploads/2022/02/2017_V-notch-report_FINAL.pdf> [Accessed 29 August 2022].

Osborne, C., & Brooks, S. A. (2006). SDS-PAGE and Western blotting to detect proteins and glycoproteins of interest in breast cancer research. In *Breast Cancer Research Protocols* (pp. 217-229). Humana Press, Totowa, NJ.

Overview of elisa (n.d.) *Thermo Fisher Scientific - UK*. Available at: https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html (Accessed: December 5, 2022).

Pandian, T. J. (1970). Ecophysiological studies on the developing eggs and embryos of the European lobster Homarus gammarus. Marine Biology, 5(2), 154-167.

Phillips. (2013). Lobsters: Biology, Management, Aquaculture and Fisheries. John Wiley & Sons, Incorporated.

Prodöhl, P. A., Jørstad, K. E., Triantafyllidis, A., Katsares, V., & Triantaphyllidis, C. (2006). European lobster-Homarus gammarus. Genetic Impact of Aquaculture Activities on Native Populations. Final Scientific Report, 91-98.

Rato, Novais, S. C., Lemos, M. F. L., Alves, L. M. F., & Leandro, S. M. (2017). Homarus gammarus (Crustacea: Decapoda) larvae under an ocean acidification scenario: responses across different levels of biological organization. Comparative Biochemistry and Physiology. Toxicology & Pharmacology, 203, 29–38. https://doi.org/10.1016/j.cbpc.2017.09.002

Ream, W., & Field, K. G. (1998). *Molecular biology techniques: an intensive laboratory course*. Academic Press.

Rotzer, & Haug, J. T. (2015). Larval Development of the European Lobster and How Small Heterochronic Shifts Lead to a More Pronounced Metamorphosis. International Journal of Zoology, 2015, 1–17. https://doi.org/10.1155/2015/345172

Rosa, R., Calado, R., Andrade, A. M., Narciso, L., & Nunes, M. L. (2005). Changes in amino acids and lipids during embryogenesis of European lobster, Homarus gammarus (Crustacea: Decapoda). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 140(2), 241-249.

Ruethers, T., Taki, A. C., Khangurha, J., Roberts, J., Buddhadasa, S., Clarke, D., ... & Koeberl, M. (2020). Commercial fish ELISA kits have a limited capacity to detect different fish species and their products. *Journal of the Science of Food and Agriculture*, *100*(12), 4353-4363.

Schmalenbach, I., & Franke, H. D. (2010). Potential impact of climate warming on the recruitment of an economically and ecologically important species, the European lobster (Homarus gammarus) at Helgoland, North Sea. Marine biology, 157(5), 1127-1135.

Sibert, V., Ouellet, P., & Brêthes, J. C. (2004). Changes in yolk total proteins and lipid components and embryonic growth rates during lobster (Homarus americanus) egg development under a simulated seasonal temperature cycle. Marine Biology, 144(6), 1075-1086.

Skerritt, D. J., Robertson, P. A., Mill, A. C., Polunin, N. V., & Fitzsimmons, C. (2015). Fine-scale movement, activity patterns and home-ranges of European lobster Homarus gammarus. Marine Ecology Progress Series, 536, 203-219.

Smith, F. X. (1999). *Bleach-dipped lobster detection technique*. NOAA report 96-NER-106.

The Fishing Daily. (2023, January 13). Cornwall fishermen plead guilty to lobster fishing offences. Retrieved from https://thefishingdaily.com/latest-news/cornwall-fishermen-plead-guilty-to-lobster-fishing-offences/

Tiu, Shirley Hiu Kwan, Ho-Lam Hui, Brian Tsukimura, Stephen S. Tobe, Jian-Guo He, and Siu-Ming Chan. "Cloning and expression study of the lobster (Homarus americanus) vitellogenin: Conservation in gene structure among decapods." General and Comparative Endocrinology 160, no. 1 (2009): 36-46.

The National Lobster Hatchery. 2022. European Lobster Biology | Biology of the Homarus gammarus. [online] Available at: <https://www.nationallobsterhatchery.co.uk/lobster-biology/> [Accessed 26 August 2022].

The Lobsters and Crawfish (Prohibition of Fishing and Landing (Amendment) (England) Order 2017. (SI 899) Available at: https://www.legislation.gov.uk/uksi/2017/899/made. (accessed 12 September 2022).

Tully, O., Roantree, V., & Robinson, M. (2001). Maturity, fecundity and reproductive potential of the European lobster (Homarus gammarus) in Ireland. Journal of the Marine Biological Association of the United Kingdom, 81(1), 61-68.

Tsukimura, B. (2001). Crustacean vitellogenesis: its role in oocyte development. American Zoologist, 41(3), 465-476.

Tsukimura, B., Waddy, S. L., Vogel, J. M., Linder, C. J., Bauer, D. K., & Borst, D. W. (2002). Characterization and quantification of yolk proteins in the lobster, Homarus americanus. Journal of Experimental Zoology, 292(4), 367-375.

Vaughan, D. (2008). Synopsis of Work Conducted to Develop a Test to Identify Lobsters (Hommarus gammarus) that Have Had Their Eggs Scrubbed Illegally. Norfolk: Eastern Sea Fisheries Joint Committee.

Vives i Batlle, J., Wilson, R. C., Watts, S. J., McDonald, P., Jones, S. R., Vives-Lynch, S. M., & Craze, A. (2010). An approach to the assessment of risk from chronic radiation to populations of European lobster, Homarus gammarus (L.). Radiation and environmental biophysics, 49(1), 67-85.

Watson, H. V., McKeown, N. J., Coscia, I., Wootton, E., & Ironside, J. E. (2016). Population genetic structure of the European lobster (Homarus gammarus) in the Irish Sea and implications for the effectiveness of the first British marine protected area. Fisheries Research, 183, 287-293.

Wilson, D. T., Vaughan, D. U. N. C. A. N., Wilson, S. K., Simon, C. N., & Lockhart, K. A. T. H. Y. (2006). A Preliminary assessment of the efficacy of a Chlorine Bleach Detection Method for use in Spiny Lobster (Panulirus argus) fisheries.