Establishing a protocol to measure tetrodotoxin (TTX) levels in small-bodied newts (genus *Lissotriton*) in western Europe

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Abstract

Tetrodotoxin (TTX) is a defence toxin most commonly found in marine organisms, and amphibians are the only land vertebrates (tetrapods) which are known to produce TTX. However, the origin of TTX in amphibians remains largely unknown, and our knowledge about the existence and distribution of TTX across taxa and populations is very incomplete. The present study summarises our knowledge of TTX in amphibians, and describes a series of experiments to determine whether TTX can be detected in newts (genus *Lissotriton* and *Icthyosaura*) across north-eastern Europe. The study was based on eggs and fresh roadkills collected in England, Scotland, Wales and France. The roadkill samples were collected at a site inhabited by both *Lissotriton vulgaris* and *L. helveticus*, and species identification was attempted using mtDNA sequencing. An initial set of TTX detection experiments considered all samples, and trialled alternative extraction protocols before employing HPLC/UV/Vis spectrometry. While strong candidate peaks for TTX were identified in some samples, the employed approach was not sufficiently sensitive to unambiguously demonstrate its presence. Three of the adult newts collected from England were further tested for TTX using liquid chromatography and high-resolution mass spectrometry. No tetrodotoxin was detected in any of the newts that were tested. The findings are discussed in light of the observed constraints by the protocols used and should serve as a useful basis for future studies on the presence of TTX in European amphibians.

Contents

1. Introduction……5

1.1 Animal Defence Mechanisms Against Predators…… ……5

1.2 Newts and Salamanders: Order Caudata…….. 8

1.2.1 Newts of the Western Palearctic.....10

1.3 Tetrodotoxin…… 14

1.4 Techniques to Identify and Quantify TTX…… 21

1.5 Aims and Objectives…… 29

2. Materials and Methods……31

2.1 Fieldwork and Sample Collection…… 31

2.2 Genetic Species Identification…… 34

2.3 TTX Detection Method Development: Sample Preparation…… 36

2.4 TTX Detection…… 38

2.4.1 TTX Standard Formation…… 38

2.4.2 HPLC/UV/Vis/MS/FLD Detection…… 39

2.4.3 LC-MS Detection……40

3. Results……42

3.1 Genetic Species Identification……42

3.2 TTX Standard……42

3.3 TTX Detection…… 45

3.4 LC-MS Analysis……54

4. Discussion..... 57

4.1 Fieldwork and Study Sites..... 57

4.2 Storage and Transportation..... 62

4.3 Sample Preparation, LC-MS and Repeat Experiments..... 63

4.4 Species Identification..... 66

4.5 Impacts of Tetrodotoxin..... 68

4.6 Further Research Opportunities..... 74

4.7 Conclusion..... 75

5. References..... 77

6. Appendix..... 89

6.1 Gels..... 89

6.2 Sample Preparation Records..... 91

6.3 HPLC/UV/Vis Results..... 93

6.4 Sequences..... 112

1. Introduction

1.1 Animal Defence Mechanisms Against Predators

Since the first organism evolved to consume another, defence mechanisms have in turn evolved so that prey organisms may evade their predators. Defence mechanisms are seen in all types of organisms, including bacteria, fungi, plants and animals, and the predator-prey relationship has led to various forms of defence mechanisms evolving in prey species. These mechanisms help prey to flee, fight, avoid detection, or they can increase chances of prey rejection. Well known mechanisms include camouflage, mimicry, poison, venom, aposematic colouration, warning signals, as well as various other fight or flight enhancements. Endler (1991) suggested six stages of predation: encounter; detection, identification, approach, subjugation, and consumption. Arbuckle (2015) pointed out that most defence mechanisms will in principle act upon one or more of these phases.

Camouflage takes various forms. It can involve either dull or bright colouration that mimics the background that the species is in (Stevens & Merilaita, 2011), or colours that mimic another species that is less likely to be attacked such as another predator, either Batesian or Müllerian (Bates, 1862, cited in Kazemi, Gamberale-Stille, Wåtz, Wiklund & Leimar, 2018; Müller, 1876). In addition, some species such as octopuses are able to change their colour and texture to match their backgrounds and also mimic shapes (Laschi, 2017). Camouflage that at first appears to make the species stand out, known as dazzle patterns, confuse the observer and cause them to misjudge size, shape, speed and direction. This type of camouflage also enables a herd to blend together, thus making it more difficult for a predator to pick out one individual, as seen in the striped pattern of the zebra (How &

Zanker, 2014). A further type of camouflage seen in many animal species involves a darker colour on the dorsal surfaces and a paler colour on the ventral surface. This is known as countershading. Light sources from above create shadows on the underside of animals which help to determine shape. A paler underside offsets this shading to confuse the onlooker (Cuthill et al., 2016). This is seen in prey such as newts, but also predators wishing to avoid detection such as tigers.

Aside from visual means of defence, the range of chemical defence mechanisms has received much debate within the scientific community. The definitions of venom and poison appear to change over time, and consequently cause ambiguity. Nelson et al. (2014) performed an extensive literature review and presented a conclusion that defines venom and poison, with the addition of a new term 'toxungens' which covers those which do not fit into either the poison or venom category. Venom is described as being deliberately delivered to a target animal via a wound. Poison is passively delivered, i.e. lacks a delivery method and is either ingested, inhaled or absorbed. The new term, 'toxungens' includes toxins that are delivered to the target animal with no accompanying wound. These defence mechanisms are well documented throughout the animal kingdom (Fry, 2015).

Toxins, as described by Vogt (1970), should adhere to the following criteria: that it is a naturally occurring substance, either an entirely foreign substance to the victim or foreign in the dosage in which it is received by the victim, and that it should have a negative impact on the victim. There are three categories of toxins: biological toxins produced by living organisms, environmental toxins occurring naturally in the environment, and anthropogenic toxins which are man-made (Nelson et al. 2014). For this study, toxin will hereafter refer to biological toxins. A species that possesses a toxin can use this as a poison or venom.

However, Nelson et al. (2014) point out it is the relative dose of the toxin which determines the harm that the toxin does. Many substances are toxic in large enough quantities and some highly toxic substances are harmless in very small quantities. They are acquired in two different manners: through biosynthesis, in which the toxin is created by the species itself, or sequestration in which the toxin is up-taken by the species from the environment (Harris & Arbuckle, 2016).

Venom is notably found in many marine species, as well as amphibians, reptiles, and insects (Harris & Arbuckle, 2016), although is also found in some mammals (Ligabue-Braun, Verli & Carlini, 2012). Some definitions of venom would also place some plant species as venomous (Maitai, Talalaj, Talalaj & Njoroge, 1981). There are numerous types of venom that are made up of either one or more toxins. The ways in which venoms are delivered are manifold, including stinging, biting, spraying, or injecting through spines or hairs. Venom is used as a defence mechanism as well as predation and occasionally intraspecific competition, whereas poison is used solely for defence (Harris & Arbuckle, 2016).

Like venoms, poisons can be made up of one or more toxins. They are common in a vast array of species, famously plants, fungi, insects, reptiles, amphibians and marine taxa. The evolution of warning signals, or aposematism, in poisonous species has facilitated the evolution of this chemical defence. Predators learn to avoid these species after unfavourable encounters whereby the prey is distasteful or toxic (Servedio, 2007). This predator learning also explains why poisonous species and palatable mimics often merge to look similar. Additionally, aposematism and camouflage are often used together, whereby the individual will expose the colourful body part when threatened, but it is otherwise

camouflaged, such as in newts, which lift their bodies off the ground and display their underside when threatened (Kupfer & Teunis, 2001).

Evolution offers creative ways in which a predator responds to defence mechanisms. For example, to deal with poison and venom, predators will develop high resistance to the toxins that are present in particular prey species (Philips & Shine, 2006). This explains why such high levels of venom are seen in prey; as the predator becomes more resistant to the toxin, the prey evolves a more potent toxin. Also, many predators have heightened non-visual senses making camouflage colours redundant, such as chemoreception and echolocation (Schwenk, 1994; Yong-Yi, Lu, Gui-Sheng, Robert, Ya-Ping, 2012). Prey species in turn evolve mechanisms in response to their predators' abilities, creating an arms race of antagonistic co-evolutions.

1.2 Newts and Salamanders: Order Caudata

The phylogenetic classification of amphibians is an ongoing process subject to change with new scientific research. However, a large study and collaboration of data sets in 2011 by Pyron and Wiens, using a combination of morphological and genetic data has provided a widely accepted classification system.

Amphibians (Amphibia), with 8285 species as of March 2021 (Amphibiaweb, 2021), include the orders frogs (Anura) caecilians (Gymnophiona) and salamanders (Caudata). The salamanders make up 740 of this number. Salamanders are tailed amphibians with an elongated body. They have limbs which sit at right angles to the body and permeable skin, allowing them to occupy aquatic or damp niches. The family *Salamandridae* contains all true

salamanders and newts. Of the family *Salamandridae*, the sub-family *Pleurodelinae* contains all 103 species of true newts (Amphibiaweb, 2020; Pyron & Wiens, 2011), despite the words 'newt' and 'salamander' often being used interchangeably.

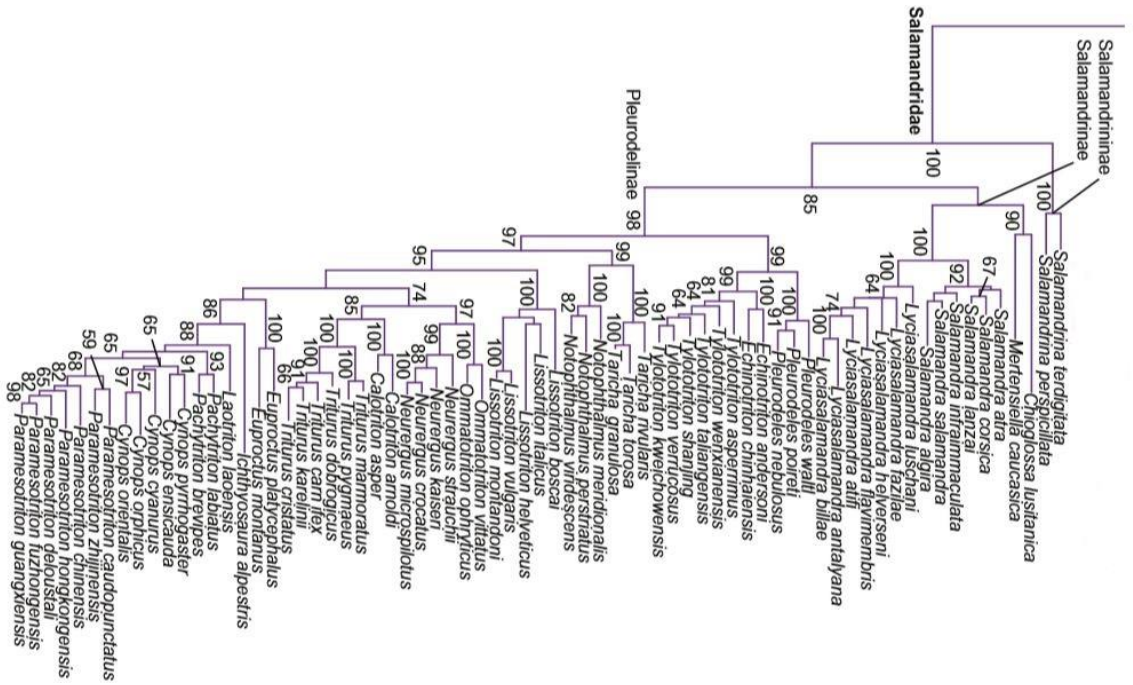


Figure 1. Phylogenetic tree showing clades of the family Salamandridae *taken from Pyron & Wiens (2011).*

Found in North America, Europe and Asia, newts have many adaptions to occupy various niches, from the temperate ponds of Britain to the forests and caves of the Middle-East. Newts are opportunistic carnivores and will prey on anything that is suitable for their current jaw size, allowing them to populate and thrive in many freshwater aquatic habitats (Griffiths, 1996).

Most newts are either fully aquatic or semi-aquatic, spending their adult lives on land and returning to water to breed. The breeding season of newts is usually in the warmer spring to summer months. Eggs are almost always laid in water, often hundreds per female during each breeding season, with each egg wrapped in aquatic vegetation for protection against

predators. Most newts spend their larval stages in water, with the tadpoles developing front legs before the back legs, unlike frogs which develop the back legs first (Griffiths, 1996). During later summer months, the juvenile newts will lose their gills and progress onto land, where they will spend their time in damp places such as under rocks, before returning to the water to breed in the next breeding season. Life span of newts varies between species. The Japanese newt *Cynops pyrrhogaster*, for example, is thought to live to at least 32 years old in a study by Sousounis et al. (2015).

1.2.1 Newts of the Western Palearctic

In Western Europe, the term 'newts' usually refer to members of the former genus *Triturus* (now comprising *Triturus, Lissotriton* and *Ichthyosaura*, see for example Speybroek et al. 2020). There are three native species of newts found in Britain: the common newt or smooth newt (*Lissotriton vulgaris*), the palmate newt (*L. helveticus*), and the great crested newt (*Triturus cristatus*). These species are listed as Least Concern on the IUCN Red List (IUCN, 2020). The alpine newt (*Ichthyosaura alpestris*) is native to central Europe and is also locally recorded in Britain. However, it is not native and considered an invasive species that likely arrived via deliberate introductions from humans (Froglife, 2020). The great crested newt is found in northern and central Europe and has a patchy distribution across the UK, not including Ireland (Jehle, Thiesmeier & Foster, 2011). *Lissotriton vulgaris* has a range across Europe, including Great Britain and Ireland. *L. helveticus* are found in most of the UK and across western parts of Europe (IUCN, 2020).

As well as the four newt species found in Britain, there are three additional species found in adjacent France. These are the Pyrenean mountain newt (*Calotriton asper*), the Corsican

mountain newt (*Euproctus montanus*), and the marbled newt (*Triturus marmoratus). E. montanus* is native only to France, whereas *C. asper* and *T. marmoratus* are distributed further across western Europe (Amphibiaweb, 2020). The IUCN (2020) lists *E. montanus* and *T. marmoratus* as Least Concern. Of all of these species in Britain and France, only C. asper is listed as Near Threatened. This study is limited to *L. vulgaris*, *L. helveticus*, and *I. alpestris*.

The appearance of *L. vulgaris* and *L. helveticus* is similar, both having smooth skin that is brown, yellow brown or grey brown in colour with black spots, with a lighter coloured belly that is a yellow or orange colour, often with spots (e.g. Griffiths, 1996) (Figure 2, 3 and 4) Adult *L. helveticus* are up to 9 cm in length, and *L. vulgaris* attain maximally 10cmThe most obviously distinguished difference between these two species is the lack of throat spots on *L. helveticus*, and during breeding some additional differences develop in the males. Male *L. helveticus* can be identified as such by a filament on the end of their tails and a smooth, low, crest that is higher on the tail (Figure 4). In contrast, male *L. vulgaris* develop a notched dorsal crest (Griffiths, 1996) (Figure 2). The dorsal surface of *I. alpestris* is similar in colour but sometimes darker or almost black in appearance. White spots can also be present that produce a mottled colour. Ventral surfaces are brighter yellow or orange than *L. vulgaris* and *L. helveticus*, and the crests in males is low, smooth and spotted (Amphibiaweb, 2020) (Figure 5). Therefore, *I. alpestris* is distinguishable from *L. vulgaris* and *L. helveticus*.

The eggs of *L. vulgaris* and *L. helveticus* are indiscernible in appearance, both being pale grey or beige in colour and approximately 3 mm in diameter (Figure 6). *I. alpestris* eggs are distinguishable from the *L. vulgaris* and *L. helveticus* eggs; they are larger and are a darker grey or brown in colour (Griffiths, 1996). These eggs are all easily distinguishable from *T. cristatus* eggs, which are larger than the *L. vulgaris* and *L. helveticus* eggs and white in colour

(Amphibian and Reptile Conservation, 2020; Holmes, 2014). *Icthyosaura alpestris each* lay up to 190 eggs per breeding season, *L. vulgaris* will lay up to 300 eggs and *L. helveticus* will lay up to 440 eggs. These newts will reach sexual maturity at 2-3 years old and live around 12-14 years, therefore will potentially lay thousands of eggs in their lifetime (Amphibiaweb, 2020). Newts are an r-selected species, and most of the eggs laid are unlikely survive until adulthood (Verrell & Francillon, 1986), mostly due to predation from other animals.

Figure 2. Male L. vulgaris *underside (S. Sivanesan, taken from Froglife, 2020)*

Figure 3. Male and female L. vulgaris *(M. Zekhuis, taken from Froglife, 2020)*

Figure 4. Male L. helveticus *(J. Howard, taken from Froglife, 2020)*

Figure 5. Male I. alpestris *(K. Marijnissen, taken from Froglife, 2020)*

Figure 6. Eggs of L. vulgaris *(K. Marijnissen, taken from Froglife, 2020)*

Lissotriton vulgaris, *L. helveticus* and *I. alpestris* differ in their habitat preferences. *Icthyosaura alpestris* is most commonly found in higher altitudes of mountainous and hilly regions, although can also live in lower altitudes (Griffiths, 1996; Winandy, Legrand & Denoël, 2017). It is often found in forested regions or areas that were previously forests. *Lissotriton vulgaris* is also commonly found in forested or previously forested areas. However, it is much more likely to be found at lower altitudes than *I. alpestris*, also thriving in areas of meadow, bushland, gardens, or agricultural land (Griffiths, 1996; Rannap, Lõhmus & Linnamägi, 2012). *Lissotriton helveticus* occupies similar habitats to *L. vulgaris*, commonly marshland and forests, as well as agricultural land. However, *L. helveticus* are more common in areas with more acidic soil as well as oligotrophic environments. Despite this, both species are able to survive in similar conditions and are frequently found living in shared habitats (Griffiths, 1996). Terrestrial habitats in which these three newts reside are always in the vicinity of bodies of water in which they can breed. These waters can be stagnant or slow moving, and range from puddles and ditches to ponds and lakes. *Icthyosaura alpestris* is more aquatic than *L. vulgaris* and *L. helveticus*. All species hibernate usually under rocks or undergrowth, until spring when the breeding season begins, at which time they return to the water (Amphibiaweb, 2020).

Newts are generally opportunistic feeders, and the study species are no exception. Being nocturnal, these species mostly hunt at night (Griffiths, 1996). Larvae prey upon microcrustaceans until they are large enough to consume various aquatic insects and molluscs. Metamorphosed adults will transition to land and consume terrestrial prey, such as insects, worms, molluscs and crustaceans (Covaciu-Marcov et al., 2010; Roşca, Gherghel, Strugariu & Zamfirescu, 2013). Additionally, these species may also display oophagy (Amphibiaweb, 2020). The eggs and aquatic larvae are commonly preyed upon by various aquatic insects, crustaceans, fish and even other newts (Miaud, 1993; Oriazola & Brana, 2003). Adult terrestrial individuals are vulnerable to predation from hedgehogs, birds, snakes and rats (Froglife, 2020).

1.3 Tetrodotoxin

Tetrodotoxin (TTX) is a highly potent neurotoxin found in a range of marine species, notably the puffer fish (fugu). TTX is also found in terrestrial species, famously in the California newt (*Taricha torosa*) (Bucciarelli, Li, Zimmer, Kats & Green, 2014). Previous research described the toxin found in this species as tarichatoxin (Brown & Mosher, 1963), although it was later shown to be TTX (Buchwald et al., 1964; Mosher, Fuhrman, Buchwald & Fischer, 1964). The toxin was first isolated by Dr. Yoshizumi Tahara in 1909 (Bane, Lehane, Dikshit, O'Riordan & Fury, 2014) and has since been proven to be over one thousand times more poisonous than cyanide (Lorentz, Stokes, Röβler & Lötters, 2016). Tetrodotoxin was first discovered in the pufferfish family *Tetrodontidae*, from where it takes its name accordingly (Williams, 2010). Several other marine taxa are known to possess the toxin in addition to the pufferfish, for example the ocean sunfish, the horseshoe crab and the blue ringed octopus (Dao, Takata,

Sato, Fukuyo, & Kodama, 2009; Saito, Noguchi, Shida, Abe, & Hashimoto, 1991; Yotsu-Yamashita, Mebs, Flachsenberger, 2007). Newts, along with frogs and flatworms, are the only terrestrial species known to possess TTX (Yotsu-Yamashita, Mebs & Yasumoto, 1992; Daly, Gusovsky, Myers, Yotsu-Yamashita & Yasumoto, 1994; Pires Jr. et al., 2005; Stokes et al., 2014). As of 2016, around 140 species were known to possess TTX (Lorentz, 2016). Table 1 shows an updated list of terrestrial species for which TTX has been recorded.

*Table 1. Table of terrestrial species known to possess tetrodotoxin, including approximate levels detected and presence of 6-epiTTX, 11-deoxyTTX and 11-oxoTTX, adapted from Hanifin (2010) with additional species from various sources. The list may not be comprehensive. *The classification of this species as a TTX bearer has been under scrutiny in the same study (Hanifin 2010)*

Order	Family, Species	Estimated levels of TTX µ / individual *µ/g	Analogues	References
Caudata	**Ambystomatidae**			
	*Ambystoma tigrinum**	12.6–17.6	6-epiTTX, 11-deoxyTTX	(Yotsu, Iorizzi, & Yasumoto, 1990)
	Salamandridae			
	Cynops ensicauda	9.6–1540	6-epiTTX, 11-deoxyTTX	(Yasumoto, Yotsu & Murata, 1988; Yotsu et al., 1990; Mosher, Fuhrman & Buchwald, 1964; Wakely, Fuhrman, Fuhrman, Fischer & Mosher, 1966)
	Cynops pyrrhogaster	8–616	6-epiTTX, 11-deoxyTTX	(Yotsu et al., 1990; Mosher et al., 1964; Tsuruda, Arakawa & Noguchi, 2001; Wakely et al., 1966; Brodie Jr, Hensel & Johnson, 1974; Tsuruda, K. et al., 2002)
	Cynops orientalis	36.25 ± 21.51*	6-epiTTX, 11-oxoTTX	(Yotsu-Yamashita, Toennes & Mebs, 2017)

Notophthalmus viridescens	9.6–220	6-epiTTX, 11-deoxyTTX	(Yotsu-Yamashita & Mebs, 2001; Yotsu-Yamashita, Mebs, Kwet & Schneider, 2007; Mosher et al., 1964; Yotsu-Yamashita & Mebs, 2003; Wakely et al., 1966; Brodie Jr et al., 1974; Levenson & Woodhull, 1979)
Paramesotriton hongkongensis	8–11		(Yotsu et al., 1990; Brodie Jr et al., 1974)
Paramesotriton chinensis	8.22 ± 7.08*	6-epiTTX, 11-oxoTTX	(Yotsu-Yamashita, Toennes & Mebs, 2017)
Taricha granulosa	<1–14,000	6-epiTTX, 11-deoxyTTX	(Cardall, Brodie Jr, Brodie III & Hanifin, 2004; Hanifin, Brodie III & Brodie Jr, 2002; Yotsu et al., 1990; Brown & Mosher, 1963; Buchwald et al., 1964; Mosher et al., 1964; Kotaki, Y. & Shimizu, 1993; Hanifin, Yotsu-Yamashita, Yasumoto, Brodie III & Brodie Jr, 1999; Wakely et al., 1966; Brodie Jr et al., 1974; Brodie Jr, 1968; Hanifin, Brodie III & Brodie Jr, 2004; Hanifin, Brodie III & Brodie Jr, 2003; Shimizu & Kobayashi, 1983; Hanifin, Brodie III & Brodie Jr, 2008)
Taricha rivularis	96–550		(Myers, 1942; Wakely et al, 1966; Brodie Jr et al., 1974)
Taricha torosa	<1–3000	6-epiTTX	(Wakely et al., 1966; Brodie Jr. et al., 1974; Shimizu & Kobayashi, 1983; Hanifin et al., 2008)
Triturus alpestris (Icthyosaura alpestris)	0–41	6-epiTTX	(Yotsu et al., 1990; Yotsu-Yamashita et al., 2007; Wakely et al., 1966)
Triturus cristatus	0–9	6-epiTTX	(Yotsu-Yamashita et al., 2007; Wakely et al., 1966)
Triturus helveticus (Lissotriton helveticus)	0–8	6-epiTTX	(Yotsu-Yamashita et al., 2007)
Triturus marmoratus	0.16–0.66		(Mosher et al., 1964; Wakely et al., 1966)
Triturus vulgaris (Lissotrition vulgaris)	0–8	6-epiTTX, 11-deoxyTTX	(Yotsu et al., 1990; Yotsu-Yamashita et al., 2007; Wakely et al., 1966)

	Pachytriton labiatus	6.25 ± 14.75*	6-epiTTX, 11-oxoTTX	(Yotsu-Yamashita, Toennes & Mebs, 2017)
	Laotriton laoensis	0.51 ± 0.48*	6-epiTTX, 11-oxoTTX	(Yotsu-Yamashita, Toennes & Mebs, 2017)
	Paramesotriton deloustali	0.10*	6-epiTTX, 11-oxoTTX	(Yotsu-Yamashita, Toennes & Mebs, 2017)
	Paramesotriton guangxiensis	0.07*	6-epiTTX, 11-oxoTTX	(Yotsu-Yamashita, Toennes & Mebs, 2017)
Anura	**Brachycephalidae**			
	Brachycephalus ephippium	<1–22.4	6-epiTTX, 11-deoxyTTX	(Pires Jr. et al., 2002; Pires et al., 2003; Pires et al., 2005; Sebben, Schwartz, Valente & Mendes, 1986)
	Brachycephalus pernix	5		(Pires et al., 2005)
	Dendrobatidae			
	Colostethus inquinalis	0.1–1.4		(Daly, Gusovsky, Myers, Yotsu-Yamashita & Yasumoto, 1994)
	Bufonidae			
	Atelopus chiriquiensis	33		(Yotsu-Yamashita & Tateki, 2010; Kim, Brown, Mosher & Fuhrman, 1975; Pavelka, 1977)
	Atelopus ignescens	<1.0–1.5		(Daly et al., 1994)
	Atelopus oxyrhynchus	32–198		(Yotsu-Yamashita, Mebs & Yasumoto, 1992; Mebs & Schmidt, 1989)
	Atelopus peruensis	3.2–4.4		(Mebs, Yotsu-Yamashita, Yasumoto, Lotters & Schluter, 1995)
	Atelopus spumarius	1.6–3.5		(Daly et al., 1994)
	Atelopus spurelli	<1–1.1		(Daly et al., 1994)
	Atelopus subornatus	3.2–17.6		(Mebs et al., 1995)
	Atelopus varius	16–26		(Yotsu-Yamashita & Tateki, 2010; Kim et al., 1975; Daly et al., 1994; Daly, Padgett, Saunders & Cover Jr, 1997)
	Rhacophoridae			
	Polypedates sp.	4.8–198		(Tanu, Mahmud, Tsuruda, Arakawa & Noguchi, 2001)

The origins of TTX in marine taxa are widely believed to be exogenous and derived from accumulation of TTX-containing bacteria via the food chain, or endosymbiotic bacteria living

within the individual (Lago et al., 2015). Various marine bacteria have been found to contain TTX (Simidu, Noguchi, Hwang, Shida & Hashimoto, 1987). Evidence for ingestion of TTX-containing bacteria comes from studies in which cultured puffer fish are shown to be non-toxic, which can be reversed when fed a diet containing TTX (Saito et al., 1984; Ji, Liu, Gong, Zhou & Wang, 2011). The origin of TTX in terrestrial species has been under scrutiny for some time. For example, Lehmen, Brodie Jr, & Brodie III (2004) suggest that an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa* is unlikely, as no amplification of bacterial DNA was found when sampling parts of the newt in which TTX was known to be present. Support for the hypothesis of origins via food chain has come from Kudo, Chiba, Konoki, Cho & Yotsu-Yomashita (2015), who discovered that the Japanese fire bellied newt (*Cynops pyrrhogaster)* possessed no TTX when reared in captivity, as opposed to the wild caught specimens which were found to contain TTX. More recently, Vaelli et al. (2020) have isolated TTX producing bacteria from the skin of toxic newts, which. in contrast to previous studies, strongly suggests that TTX in newts does have a bacterial origin.

Tetrodotoxin has the formula $C_{11}H_{17}N_3O_8$ and the chemical structure is shown in Figure 7. The toxin takes effect by binding to voltage gated sodium channels. This prohibits sodium ions (Na+) from moving across cell membranes and so blocks propagation of action potentials, leading to the inhibition of nerve impulses.

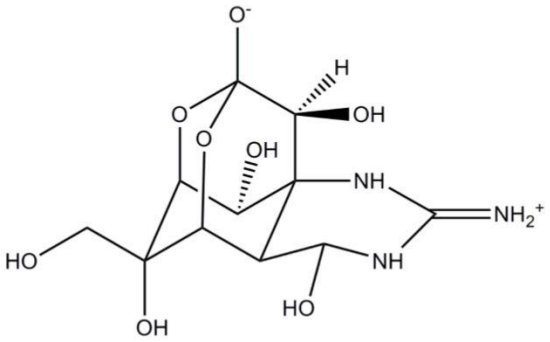


Figure 7. Chemical structure of tetrodotoxin (taken from Lago, Rodríguez, Blanco, Vieites & Cabado, 2015)

TTX has around 28 known analogues (Tamele, Silva & Vasconcelos, 2019) including the chemical equilibrium analogues, 4-*epi*TTX and 4,9 anhydroTTX (Figure 8).

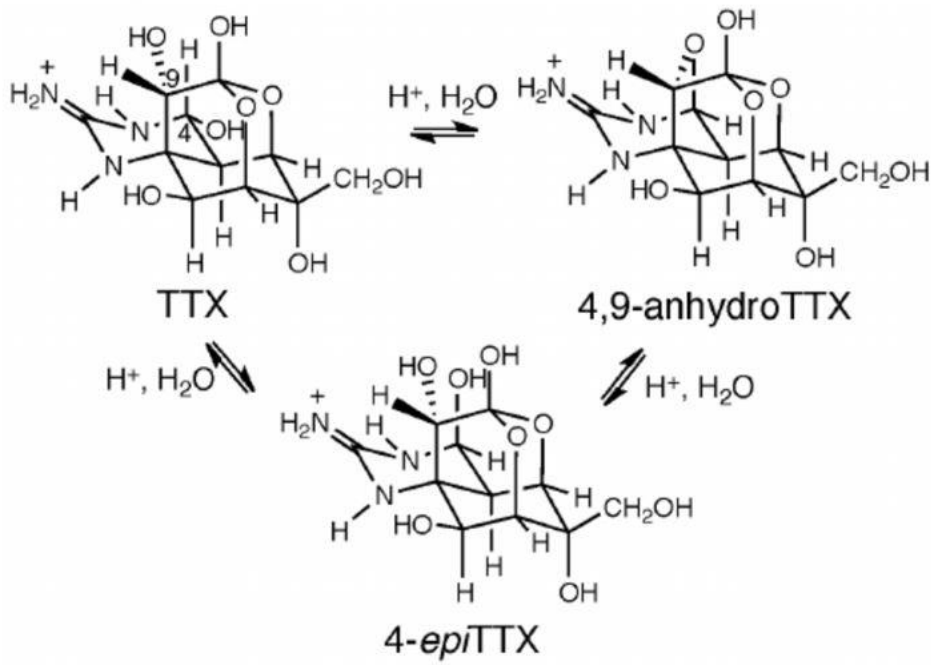


Figure 8. TTX and its analogues 4-epiTTX and 4,9-anhydroTTX (taken from Teramoto & Yotsu-Yamashita, 2015)

Research suggests the toxin is up-taken via the food chain by a prey species, which is then in turn eaten by predator species. The predator then becomes poisoned with usually fatal

results. Despite the extreme levels of toxicity, species that possess TTX are still eaten by humans. Puffer fish are considered a delicacy in Japan and a licence is required by chefs preparing the fish for consumption, due to the dangers of TTX (Stommel & Watters, 2004). TTX is found in high concentrations in the skin, ovaries and liver which therefore must be removed from the fish before consumption (Bane et al., 2014).

Symptoms of TTX poisoning in humans vary depending on the amount that has been ingested. In very small doses, TTX has been experimented with as a painkiller in cancer patients, due to its nerve blocking qualities (L'Abbee, 2003). However, even mild cases of TTX poisoning can cause gastrointestinal issues, including nausea and vomiting. In most cases, early symptoms begin within 45 minutes and include perioral numbness and paraesthesia. These then progress onto paralysis and respiratory failure within several hours. Within 24 hours, a loss of consciousness will occur, respiratory failure becomes severe enough for hypoxia to occur and hypotension, bradycardia, and cardiac dysrhythmias may also present (Isbister & Kiernan, 2005). TTX can also enter the body via inhalation, injection or through abraded skin (Lorentz, 2016).

With respect to predator-prey interactions involving TTX, a main model system is based on the relationship between rough-skinned newts (*Taricha granulosa*) and garter snakes (*Thamnophis sirtalis*). *Thamnophis sirtalis* have developed a resistance to TTX, and in turn newts have developed higher levels of TTX in a prime example of an evolutionary arms race. Due to the feeding method of snakes, dissection of prey to avoid toxic parts is not possible. Consequently, these snakes have evolved new physiological and behavioural mechanisms to deal with their prey (Williams, Hanifin, Brodie Jr & Brodie II, 2012). In *T. sirtalis*, the binding of the TTX is hindered by structural differences in the sodium channels (Geffeney, Fujimoto,

Brodie III, Brodie Jr & Ruben, 2005), allowing them to prey upon the newts. In addition, *L. sirtalis* are able to detect TTX concentrations in an individual newt upon the initial strike, and will reject the newt if concentrations are higher than the snake can tolerate (Williams, Hanifin, Brodie Jr & Brodie II, 2010). Tasting and rejection creates a fitness advantage to a lethal level of a toxin, solving the paradox whereby both predator and prey are killed upon interaction (Skelhorn & Rowe, 2006). This, in turn, increases the Darwinian fitness of newts with higher concentrations of TTX, creating the arms race between the two species. As well as the ability of *T. sirtalis* to detect the levels of toxin, there is evidence that *T. granulosa* are able to detect the kairomone released when *T. sirtalis* has successfully consumed a toxic newt (Gall, Farr, Engel & Brodie Jr, 2011). This allows the newt to differentiate between toxin-resistant and toxin-sensitive individual snakes in a given area. In addition to the detection of kairomones released by *T. sirtalis*, Bucciarelli, Green, Schaffer & Kats (2016) also suggest that the related California newts (*T. torosa*) are able to detect levels of TTX in conspecifics as well as in themselves, allowing individuals to make a choice regarding possible breeding sites.

Thamnophis. sirtalis are not alone in their ability to withstand prey containing TTX. Caddis fly (*Trichoptera*) larvae are also known to prey upon newt eggs. A study by Gall, Brodie III & Brodie Jr (2011) highlighted that larvae that preyed upon newt eggs containing TTX showed no growth differences to those that had had a diet of detritus containing no TTX.

1.4 Techniques to Identify and Quantify TTX

Various techniques have been used to detect and quantify TTX. These include assays which are based on a range of different approaches such as mouse bioassays, cell bioassays,

immunobioassays, and enzyme-linked immunosorbent assays (ELISA). Chromatography is also widely used, encompassing column chromatography, thin layer chromatography, high performance liquid chromatography (HPLC), gas chromatography (GC), and liquid chromatography (LC). Mass spectrometry (MS) is often used in tandem with these methods.

The traditional method used to determine TTX concentrations is the mouse bioassay. The protocol involves injecting a mouse with the sample mixed with acetic acid, and recording the time taken from the end of injection to the last gasp of breath. The extract is diluted until the median death time is 7-13 minutes. Toxicity is calculated in mouse units (MU) by the dose to death time relationship. 1 MU is defined as the amount of TTX required to kill a 20 g mouse in 30 minutes, which is approximately 0.22 μg (Suzuki, 2016). Whilst the mouse bioassay is a relatively cheap and effective method, it has been criticised as unethical as well as producing a number of false positives (Bodero et al., 2018; Campbell, Vilariño, Botana & Elliott, 2011). More sensitive methods are available to detect lower concentrations of toxins (Hamasaki, Kogure & Ohwada, 1996).

The use of tissue culture bioassays to detect TTX involves analysing the relative number of living cells in an in vitro environment, after being exposed to oubane and veritradine, which kills the mouse neuroblastoma cells that are used. However, if TTX is also present, the cells are protected and survive. Therefore, by counting the remaining living cells, the presence and abundance of TTX can be observed. It has been suggested as an alternative to the mouse bioassay for its simplicity and inexpensive design (Kogure, Tamplin, Simidu & Colwell, 1988). Hamasaki, Kogure & Ohwada (1995) discuss the need for improvement of this method, mainly its subjectivity, before its use as an alternative to the mouse bioassay. The method requires the morphological changes to the cells to be observed, to determine

whether they were living or dead. This is time consuming and may vary between observers and experiments. Gallacher and Birkbeck (1992) improved the subjectivity of this method by introducing a microtire plate reader, which was able to quantify the number of living cells by their uptake of vital dye Neutral red. Jellet et al. (1992) used the same method of tissue culture bioassay along with a microplate reader and cell staining and compared their results to those of mouse bioassay and HPLC. They determined that the tissue culture bioassay yielded almost identical results compared to the mouse bioassay, with more sensitive detection. However, the HPLC results were not consistent with the results of the bioassay methods. Hamasaki et al. (1996) suggested that this method was still subjective as it required a level of skill in the washing stages which remove the free dye and dead cells. They developed the method described by Manger, Leja, Lee, Hungerford & Wekell (1993) using tetrazolium salt 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), by switching for the water-soluble tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1), which eliminates the solubilization stage. The use of WST-1 tissue culture bioassay was 100 times more sensitive in the detection of TTX than the mouse bioassay, and enabled large numbers of samples to be analysed more quickly.

Ling et al. (2015) discuss the practicality, expense and precision of the traditional method of mouse bioassay as well as HPLC and other chromatography methods. They suggest that immunoassays are more economical and rapid at given precision. Immunoassays are biochemical tests for the presence of a molecule, either by testing for an antigen directly or indirectly testing for the antigen antibodies (Actor, 2012). Enzyme-linked immunosorbent assays (ELISA), a form of immunoassay, have been used to detect TTX with a limit of detection (LOD) of 5 ng/mL (Tau et al. 2010, cited in Ling et al. 2015) and 10 ng/mL (Stokes

at al. 2012, cited in Ling et al. 2015). Following on from Zhou et al. (2010) in which a colloidal gold probe was used which detected TTX in less than 10 minutes with a LOD of 40 ng/mL, Ling et al. (2015) also conducted a study using these methods to successfully detect TTX. Stokes, Williams & French (2012) agree that immunoassays are more efficient than HPLC at quantifying tetrodotoxin. They state that competitive inhibition enzymatic immunoassays (CIEIA) have been used, although the revealed findings are not repeatable without in depth knowledge of competitive enzyme immunoassays (EIA). They present a modified CIEIA method which uses monoclonal antibodies and cheaper and more readily available lab equipment, producing a LOD of 10ng/mL.

Developed in its simplest form in 1900 to separate colours, chromatography has become a successful and important method for separating compounds. Chromatography involves two stages: the stationary phase, and the mobile phase. The stationary phase consists of a solid structure such as a column that may contain a liquid. The mobile phase is the liquid or gas that flows through the stationary phase. The sample to be analysed is passed through the stationary phase, using the mobile phase, resulting in the separation of molecules. This is due to the way the separate molecules in the sample interact with the stationary phase, meaning the time at which they emerge differs (Wixom, Gehrke, Berezkin & Janak, 2010).

Using paper as the stationary phase and a liquid solvent such as water along with capillary action as the mobile phase, one of the simplest forms of chromatography, paper chromatography, is able to separate colours such as those found in ink (Du Toit, Eggen, Kvittingen, Partali & Schmid, 2012). In a more sophisticated set up, thin layer chromatography (TLC) uses a plate of either plastic, metal or aluminium foil instead of paper. This is coated with an adsorbant material such as silica gel as opposed to water

(Santiago & Strobel, 2013). Column chromatography uses the same principles but on a larger scale with a vertical column made of glass (Jesus, Ferreira, Maciel & Filho, 2019).

Developing methods from thin layer chromatography and column chromatography has led to new chromatography techniques such as high-pressure liquid chromatography (HPLC), gas chromatography (GC) and immunoaffinity chromatography which have been used in TTX detection studies. These are often used in conjunction with other methods including fluorescence detection (HPLC-FLD) and ultraviolet detection (HPLC-UV), gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) (Asakawa, Ito & Kajihara, 2013; Chen et al. 2011; Chulanetra et al. 2011; Yan, Yu & Li, 2005).

Similar to thin layer chromatography, HPLC involves the separation of substances. The liquid sample is forced through a column containing an adsorbent material at a high pressure. As each component interacts differently with the adsorbant material inside the column, the components separate and the time it takes for each component to pass through the column can be recorded. This is known as retention time. To determine specific molecule retention times, a standard is run which contains varying concentrations of the specific molecule. Results are recorded as peaks on a graph, representing retention time as well as concentration. These times can be compared to known retention times of a pure form of a substance, indicating the presence or lack of a particular analyte. This method presents an effective way of detecting and measuring the amount of TTX in a sample. When HPLC has been used to detect TTX, it has usually been supplemented by other methods as shown by Lin et al. (2014), where HPLC is shown to be a successful method to detect TTX and the analogue anhydro-TTX in gastropods. However, further analysis using mouse bioassay and

LC-MS/MS was conducted, suggesting that a combination of methods is more accurate. This approach is widely used in recent years (Liung, Fong & Tsoi, 2011; Saito et al. 2014; Indumathi & Khora, 2017).

Gas chromatography is often used as an efficient method of separating compounds in gases and particularly in volatile liquids. Furthermore, GC comprises a stationary phase and a mobile phase, where a gas such as helium or nitrogen is used for the mobile phase as opposed to a liquid. When coupled with mass spectrometry, GS-MS has been successfully used in the detection of TTX (Suenaga & Kotoku, 1980; Man, Noor, Harn, Lajis & Mohamad, 2010).

To gain usable results from chromatography, retention times must be matched with the known retention times of the substance in question. Mass spectrometry provides a useful addition to chromatography, as it enhances the detection and provides qualitative information. Mass spectrometry involves measuring the mass to charge ratio of ions. This can be used to calculate the molecular weight of molecules in a sample, allowing unknown compounds to be identified and the concentrations of known compounds to be determined. Molecules are ionised (usually positively) so that they can be deflected by a magnetic field. The molecules are then sent through a vacuum in the mass spectrometer and the deflection creates a measurable curve. The speed at which the particles were traveling is known, along with the deflection curve, and these are used to calculate the mass of the molecules. The lighter and more positively charged the ion, the greater the deflection (Gross, 2004). High resolution mass spectrometry (HR-MS) is able to measure the exact mass of analytes. It is able to measure the atomic mass of molecules to the nearest 0.001, whereas traditional MS can only measure single digit units. There are three types of instrument used; Time of flight

(TOF), orbitrap and Fourier transform ion cyclotron resonance (FTICR). High resolution mass spectrometry coupled with LC has successfully been used to detect TTX (Shalabai et al., 2016).

Man et al. (2010) point out that LC-MS is expensive, and a high level of maintenance in the machinery is needed, whereas GC-MS is a cheaper alternative. They do, however, state that LC-MS is the more effective method. Bane et al. (2014) also agree that LC-MS is the best option for detection of TTX. They argue that as TTX is non-volatile, in order to use GS, TTX first needs to be converted to a volatile derivative, which is time consuming and difficult to reproduce. Furthermore, this method requires a large amount of a sample, and is therefore problematic for the study of the small species such as *L. vulgaris* and *L. helveticus* used for this research. In addition to MS detection, ultraviolet/visible (UV/Vis) and fluorescence (FLD) spectrophotometry detection methods have been used in conjunction with chromatography. Much like MS, UV/Vis and FLD improve the accuracy of detection and the quantification of compounds.

Measuring the absorption of light, UV/Vis spectrometry uses the wave lengths of 180nm to 390nm, which encompasses the near UV range, and 390nm to 780nm, the visible range (Worsfold, 2019). Typically, in UV/Vis an electron is excited so that it becomes promoted from the highest energy occupied molecular orbital (HOMO) to the lowest energy unoccupied molecular orbital (LUMO). The smaller the energy difference between these two, the longer the wavelength absorbed by a compound. The wavelength which is absorbed the most is known as the lambda max (λ_{max}). Different molecules have difference absorption ranges, which allows them to be distinguished from other molecules within a

particular sample. Worsfold (2019) points out that UV/Vis is widely used for the quantification of molecules, although it is not commonly used for identification purposes.

Fluorescence detection (FLD) uses similar principles to UV/Vis but instead of measuring absorption of light, FLD is based on measuring light emitted. A molecule can be excited by light absorption and will then emit this back out, usually as a longer wavelength due to the lower energy. This phenomenon can happen naturally or can be artificially induced, and is known as fluorescence. Fluorescence detection is performed using a fluorometer, which usually involves using a single wavelength to excite the molecule being analysed, with a single wavelength being emitted. This method can identify and quantify molecules as low as 1 ppt. This is useful when trying to detect and compare trace amounts of a substance.

Previous methods to detect TTX and similar toxins in newts have involved killing the newts (Wakely et al., 1966; Levenson & Woodhull, 1979). Skin punches have been used as an alternative to this destructive sampling method. Johnson et al. (2018) and Hanifin et al. (2002) used a 5 mm diameter skin biopsy punch to extract a section of dorsal skin from *T. torosa* and *T. granulosa*. Bucciarelli, Li, Kats & Green (2014) develop an approach that uses a much smaller sample size of 2 mm of dorsal skin on the California newt, causing relatively little harm not impeding continued survival. As TTX is found in the skin of newts, this method is however only appropriate for larger bodied newts used, such as *T. torosa* or *T. granulosa*. *Lissotriton vulgaris* and *L. helveticus* are small bodied and would likely not withstand losing the amount of skin that is needed in order to successfully detect TTX using these methods.

1.5 Aims and Objectives

This study aims to determine if British and French populations of small bodied newts contain TTX and if so, to measure these levels. The study will seek to develop a method that provides an accurate and precise result for measuring low quantities of TTX in newt species. This will be achieved using detection methods based on UV/Vis, MS and FLD techniques. If not TTX is detected, more sensitive LC-MS is available and will be trialled. The study further aims to establish an accurate sample preparation method, which will be ascertained by the preliminary sample preparation tests. These tests will include the use of different body parts from the road kill newts which will allow for comparison of TTX levels in various locations on the newt, to determine where the concentration is highest. These levels can additionally be compared to TTX concentrations within the eggs to determine whether eggs are a suitable tissue sample for future use.

Eggs will be collected from various sites across Britain, including Wales, Scotland, England, as well as from France, comprising *L. helveticus*, *L. vulgaris*, or *I. alpestris*. Road kill newts will also be collected from a site in West Yorkshire. Due to the nature of the reproduction of newts and the abundance of these species, the collection of eggs will have a minimal impact on the ecology of these populations. This allows the comparison of TTX levels in populations of different geographical locations, as well as potentially different predation levels. The study also aims to test the hypothesis that samples taken from further north will have less TTX than those closer to glacial refugia.

Additionally, this study aims to use genetic analysis to identify the species of newts that have been sampled. If TTX is present, the species identification of each sample may then be used for comparison with other studies that have found TTX to be present.

The specific objectives are to: (1) use genetic techniques to distinguish between *L. vulgaris* and *L. helveticus* at sites where both species occur and when morphological identification is impossible; (2) establish techniques to detect TTX from skin tissue and eggs of small-bodied newts, and (3) test the available samples for the presence of TTX.

2. Materials and Methods

2.1 Fieldwork and Sample Collection

A total of approximately 200 eggs were collected from France, Wales, England and Scotland, and a total of 14 roadkill adult newts were collected from England (see Table 2).

The eggs obtained from France were collected from three ponds in the Department of Mayenne, Western France in April 2018. The three sites represented ponds 222, 232 and 2C8 in Jehle et al. (2005). Eggs were individually stored in 1 ml of acetonitrile in Eppendorf tubes and transported to Salford where they were frozen at -20 ^{o}C until sample preparation took place.

Collection of eggs from England took place in Egerton Quarry in Bolton, in May 2018. Ten eggs were collected and stored together in an Eppendorf tube, fully submerged in water from the pond in which they were collected. Eggs were transported to the laboratory with ice packs, killed by freezing within one hour and stored in a freezer until sample preparation took place.

The Scottish sites consisted of three rural and three urban ponds, part of a study system around Inverness described in Miro et al. (2017) and Rae et al. (2019). From each pond, approximately ten eggs were collected. Eggs from each site were stored in an Eppendorf tubes together in the water from the pond they were collected from. These were transported to Salford and stored in a refrigerator at 4 ^{o}C. Eggs were killed by submerging in 1 ml of 0.1M acetic acid and transferred to individual Eppendorf tubes.

Seven eggs were collected from Wales from one site. These were stored all together in the pond water in an Eppendorf tube and transported to Salford, where they were killed by freezing.

Fourteen adult newts were collected from Todmorden in West Yorkshire, in May 2018. These were all road kill and were collected at dusk. A stretch of road on which newts were known to cross during breeding season was patrolled. Newts were recently deceased, with a maximum of approximately one hour before collection, and therefore labelled as fresh. Some newts were found to have been killed before the patrol started, therefore were thought to have been killed from the previous night. Due to high levels of daytime predation, it is unlikely that any newts were over 24hrs hours old. Nevertheless, these newts were labelled as dried, as they had become dried from the daytime sun. These were transported with icepacks, and stored in a freezer within one hour of being collected.

Due to previous research on the species of newt in each site, eggs were assigned a species based on local species occurrences. Eggs from France, Scotland and England were all classed as *L. helveticus*. Eggs from Wales were classed as *I. alpestris* based on their morphology. Adult newts were tentatively identified by morphological traits. Newts with a pale throat with spots were classed as *L. vulgaris*, whilst newts with no throat spots were classed as *L. helveticus*. For a summary of collected samples see Table 2 and 3.

Table 2. Eggs collected from various locations with predicted species based on species known to reside in each location

Sample Number	Number of Eggs	Predicted Species	Location
E1	10	*L. helveticus*	Egerton Quarry, England
E2	11	*L. helveticus*	Inverness, Scotland
E3	6	*L. helveticus*	Inverness, Scotland
E4	7	*L. helveticus*	Inverness, Scotland
E5	9	*L. helveticus*	Inverness, Scotland
E6	9	*L. helveticus*	Inverness, Scotland
E7	5	*L. helveticus*	Inverness, Scotland
E8	7	*L. helveticus*	Inverness, Scotland
E9	8	*L. helveticus*	Inverness, Scotland
E10	12	*I. alpestris*	Morfa Dyffryn, Wales
E11	10	*L. helveticus*	Mayenne, France
E12	10	*L. helveticus*	Mayenne, France
E13	8	*L. helveticus*	Mayenne, France

Table 3. Newt individuals collected from Todmorden, England, with predicted species based off visual identifications

Newt Number	Predicted Species
N1	*L. helveticus*
N2	*L. helveticus*
N3	*L. vulgaris*
N4	*L. helveticus*
N5	No data
N6	No data
N7	No data
N8	*L. helveticus*
N9	*L. helveticus*
N10	*L. helveticus*
N11	*L. vulgaris*
N12	*L. helveticus*
N13	*L. helveticus*
N14	*L. vulgaris*

2.2 Genetic Species Identification

Eggs were taken from ponds in which only one species was known to reside based on known local distributions of small-bodied newt species. Roadkill newts were taken from roads which were nearby multiple known breeding ponds and therefore could not be associated with any specific pond. In order to correctly identify these individuals, genetic analysis was performed.

DNA was extracted using a QIAGEN DNEasy Blood and Tissue Kit. Approximately 25 mg of tissue was cut from each newt using a scalpel, and added to 1.5 ml Eppendorf tubes. 180 µl of buffer ATL and 30 µl of proteinase K were added, and the samples were vortexed. The Eppendorf tubes were then placed in a water bath at 56 ˚C for three hours, with vortexing at one hour intervals, until DNA was completely lysed. 200 µl of buffer AL was added and mixed by vortexing, followed by adding 200 µl of ethanol (100%) which was mixed by vortexing. The mixture was then transferred using a pipette to spin columns placed in 2 ml collection tubes. The samples were then centrifuged at 8000 rpm for one minute. The collection tubes and flow through were discarded, and the spin columns were placed inside new collection tubes. 500 µl of buffer AW1 was added and samples were centrifuged at 8000 rpm for 1 minute. The collection tubes and flow through were discarded, and the spin columns were placed in new collections tubes. 500 µl of buffer AW2 was added and samples centrifuged for a further 3 minutes at 13,400 rpm. The collection tubes and flow through were discarded again, and the spin columns were placed in 1.5 ml Eppendorf tubes. 200 µl of buffer AE was added to the spin columns to elute the DNA. After incubating the samples for 1 minute at 19 ˚C, the samples were centrifuged for 1 minute at 8000 rpm.

DNA samples were then run on an agarose gel using electrophoresis to determine quantity and quality of DNA before PCR. Gel was made using 1.5 g of agarose in 100 ml of 1 x TBE buffer. GelRed was added at a ratio of 1 µl to every 10 ml of TBE. TBE was measured using a 50 ml graduated cylinder and transferred to a conical flask, to which the agarose powder was added. GelRed was added using a pipette. The solution was microwaved in an 800 W Samsung M1719N microwave until the agarose powder had completely dissolved. This was then left to cool until safe to touch by hand. After cooling, the solution was poured into a gel rack with combs and left to set.

DNA (5 µl) was mixed with 15 µl loading buffer and loaded into each well using a pipette. The gel rack was transferred to an electrophoresis chamber and connected to a powerpack. The gel was run using electrophoresis for approximately 40 minutes at 70 V. Once complete, the gel was placed under a transilluminator and viewed using GeneSnap. Samples 4, 5 and 7 showed no DNA after initial PCRs (Appendix 1) and so the extraction of DNA from these samples were repeated.

PCRs were prepared using a mastermix composed of 1 µl of 10 x reaction buffer, 4.6 µl of PCR water, 0.3 µl of 50 mM MgCl2, 1 µl of 2mM dNTPs, 0.05 µl of taq, and 1 µl of forward and reverse primer each. These concentrations amounted to 9 µl, to which 1 µl of DNA was added to make up 10 µl reaction volumes. An approximately 800 bp long fragment of the partial ND2 mitochondrial region was amplified using PCR primers described in Babik et al. (2005; Forward: TCGAACCTACCCTGAGGAGAT; Reverse: AGGTGTGCAATGGATGAGTATG). The PCR reaction was carried out in VeritiTM 96-well thermal cyclers (Applied BiosystemsTM, UK) using the following amplification conditions: 2 min at 96 °C, followed by

37 cycles of 30 s at 94 °C, 45 s annealing at 53 °C and 1 min 30 s at 72 °C and finally 5 mins at 72 °C. The PCR reactions were carried out.

Following PCR, samples were run on a gel using electrophoresis as described above. A hyperladder (Bioline Reagents Ltd.) was loaded into the first well of each row. Gels were placed in an electrophoresis chamber and connected to a powerpack. Powerpacks were set at 70 V and were run for 30-40 minutes.

When viewed under a transilluminator, the results showed faint PCR bands and a very strong primer flare (Appendix 1-3). Therefore, the DNA was diluted in the ratio 1:10 with single distilled water. The process was repeated with the diluted samples, using double the amounts of reagents and DNA to make up a 20 µl reaction volume, which produced clearer results for some samples. As the majority of samples produced only faint bands, each sample that had produced a PCR product was merged with its corresponding sample from the various PCR runs, in order to maximise the total volume. These PCR products were sent off for commercial sequencing with the company Macrogen.

2.3 TTX Detection Method Development: Sample Preparation

Adult newts were left to defrost fully at 25 ^{o}C (room temperature) before preparation. Newts were dissected using a scalpel to remove a section of skin (~1-3 mm^2 surface area) from the tail tip, the tail base, the ventral and dorsal side, and from the fore- and hind-limb. Tail tip, as well as hand and foot sections were whole and included bone tissue. Each section was individually weighed. Tissue samples were then transferred to an Eppendorf tube and 1 ml of acetonitrile was added using a pipette. Samples were divided into groups of four,

and transferred to a sonic bath. Samples in Group 1 were run at room temperature for 15 minutes, and samples in Group 2 were run at room temperature for 30 minutes. Group 3 was run at 50 ˚C for 15 minutes, and Group 4 was run at 50 ˚C for 30 minutes. Each group contained all variants of tissue. Samples were then transferred into a syringe and filtered with 30 mm 0.4 µm nylon filters into a glass vial. Negative controls were added for each group, containing 1 ml of acetonitrile. A summary of this can be seen in Appendix 4.

The samples were analysed using HPLC/UV/Vis spectrometry, using a cell volume of 10 µl and the maximal lambda for TTX as wavelength. The results revealed no indication of TTX in the tissue samples, as the chromatograph did not reveal any bands that were absent in the negative controls (Appendix 6), indicating that the acetonitrile had not been successful in extracting the compounds from the tissue. Therefore, based on the approach by Johnson et al. (2018) and Yotsu-Yamashita, Toennes & Mebs (2017) the experiment was repeated by using 1.5 µl 0.1 M acetic acid in place of the sonication and acetonitrile, and by crushing the tissue samples in an agate pestle (~1cm diameter) and mortar (~4cm diameter) before placing in an 1.5 mm Eppendorf tube and centrifuging for 30 minutes at 13,300 RPM. Once tissue had been extracted for each sample, the remaining newt was placed in 0.1 M acetic acid and a sonic probe was used to macerate the tissue. These samples (47-50) were then centrifuged in addition to the previous samples. A summary for these samples is shown in Appendix 5.

The results from this method (Appendix 7) show that the acetic acid digestion was successful in breaking down the tissue. In addition, they indicate that the tissue types of dorsal, ventral and tail skin were efficient tissue samples for detection of compounds using the HPLC/UV/Vis spectrometer. The results from the further sonication using the sonic

probe did not provide adequate evidence that use of the sonic probe was needed, as the acetic acid alone was successful in breaking down the tissue. Samples were also run using Liquid Chromatography Mass Spectrometry (LC/MS). At 100 parts per million, the mass spectrometry did not appear sensitive enough to detect molecules in the samples and produced no clear results.

A TTX standard was prepared and run on the HPLC/UV/Vis in concentrations ranging from 1 ppm to 100 ppm. The results showed that the equipment was not sensitive enough to detect the lower amounts of TTX, as nothing was detected at 0.1%, 0.5% and 1% of stock solution. As it was expected that any levels of TTX found in this species of newt would be low, more sensitive equipment was adopted. Fluorescence detection was used, which allowed the wavelength that TTX fluoresces at to be targeted (excitation wavelength of 384 nm and emission wavelength of 505 nm), thus producing more accurate results for a lower level of the toxin.

2.4 TTX Detection

2.4.1 TTX Standard Formation

TTX was purchased from a commercial source (Sigma Aldrich item number T8024) and was stored at room temperature. The standard was prepared using 0.1 M acetic acid and run using HPLC/UV/Vis after 24 hours of arrival. Concentrations of 1 ppm to 100 ppm TTX were run three times each, and the average taken. The findings from the HPLC/UV/Vis detection showed that this method was not sensitive enough for the lower levels of TTX (Appendix 7). Therefore, fluorescence detection was adopted as a more accurate method. The

fluorometer provided consistent results for each concentration of TTX (Table 4), and this method was therefore used to run each sample.

The data were analysed using Microsoft Excel. The TTX standard data were used to create a graph showing a concentration gradient. The gradient was assessed by a linear regression, considering R^2 as a measure for goodness of fit. This could then be compared with the results from the samples to determine any concentrations of TTX using the area of the peak as a percentage against the initial mass of tissue or egg that was used.

Some samples were not run immediately, and it was hypothesised that the TTX may degrade or change whilst left at room temperature and mixed with acetic acid. Therefore, the standard was run again after approximately one month at room temperature and one week of being mixed with acetic acid. This showed different results to the initial run, with the peak retention time at around 1 minute later than previous runs. The results of this additional standard are shown in Table 5.

2.4.2 HPLC/UV/Vis/MS/FLD Detection

Newts were left to defrost fully, at 25 ^{o}C (room temperature), before preparation. One tissue sample per newt was taken from either dorsal skin, ventral skin or tail base skin and weighed. Samples were then added individually to an agate pestle and mortar and 1.5 ml of 0.1 M acetic acid was added using a pipette. The tissue was then crushed and samples were transferred to individual 1.5 mm Eppendorf tubes and centrifuged for 30 minutes at 13,300 RPM. Samples were then individually transferred to a syringe and filtered through 30mm

0.45 µl nylon filters, into glass vials. Negative controls were added for each site, containing 1.5 ml of acetic acid, which were centrifuged and filtered at the same time.

Eggs that were taken from the same site were sampled all together, totalling approximately ten eggs per sample. Eggs were left to defrost fully and dried at room temperature and all eggs from one site were weighed together. The eggs were then transferred to an agate pestle and mortar and crushed. 1.5 ml of acetic acid was then added using a pipette. The sample was then transferred to a 1.5 mm Eppendorf tube and centrifuged for 30 minutes. Samples were then removed and transferred to a syringe and filtered. Negative controls were added for each site, containing 1.5 ml of acetic acid, which were centrifuged and filtered at the same time.

A modified set up including HPLC/UV/Vis/MS/FLD was used to produce the final results. An auto injector was used to inject the samples. The sample passed through a Hichrom C18 column, which flowed into an Agilent 1200 UV/Vis excitation wavelength 384nm. The eluent was mixed with 4M NaOH and heated to 95 ^{o}C in a mixing chamber. The solution then passed through the Agilent FLD unit (excitation 384 nm emission 505 nm) before being directed to the waste.

2.4.3 LC-MS Detection

As the results from the HPLC/UV/VIS experiments proved promising but not fully conclusive (see below), three of the samples which provided the closest match (N10, N11 and N12) were taken to the Manchester Metropolitan University and run again on a more sensitive LC-MS machine. The newts from which samples showing the highest likelihood of containing

TTX were used to prepare fresh samples using the same method. These were also stored in the freezer until analysis and were run within 1 hour of being removed from the freezer.

Sections of dorsal skin from roadkill samples were removed using a scalpel, from each newt. Skin weights were 0.06 g (N10), 0.05 g (N11 and N12). 0.9 ml of acetic acid was diluted to 0.1 M and added to the newt tissue which was crushed using an agate pestle and mortar. The liquid phase was transferred to an Eppendorf tube using a pipette. 0.9 ml of acetic acid was added to an Eppendorf tube as a negative control. All samples plus the negative control were centrifuged for 30 minutes at 13,300 RPM. Samples were then filtered through 30mm 0.45µl nylon filters using a syringe, into glass vials. Samples were stored in a freezer at -20 ^{o}C, and in the fridge at 4 ^{o}C, when they were collected for analysis approximately one month later, when the samples had developed a cloudy colouration. Therefore, these samples were discarded and new samples were prepared using the same methods. These samples were stored in the freezer until analysis, and run within one hour of being removed from the freezer. Due to limitations of equipment and budget, only three samples were analysed. These three newt samples were taken to the University of Manchester and run on an LC-MS machine. The make and model for the HPLC analysis were Agilent, 1260 Infinity Series HPLC. The samples were analysed by High Resolution (HR-MS) using Time of flight (TOF) LC-MS TOF. For the MS analysis, the make and model were 6540 LC-MS QTOF. The electrospray ionisation (ESI) was run in positive mode (+), looking for the accurate mass (Monoisotopic) of TTX with an additional Hydrogen (Mass of 1.0078) to give the [M+H] ion. The solvents used for mobile phase A were UP H_2O and 0.1 % formic acid, and for mobile phase B, methanol and 0.1 % formic acid were used. Injection volumes of 20 µl were used.

3. Results

3.1 Genetic Species Identification

Many of the sequences were of poor quality and provided accurate results for only four out of the twelve newts that were sampled. These four were samples N5, N10, N11 and N13 and were categorised as *L. helveticus*. Blast was unable to find a significant match for five of the newts and the remaining three were classified as bacteria (family Xanthomonadaceae) suggesting contamination during PCRs or bacterial colonisation of samples. The samples that were classified as *L. helveticus* were matched to sequences available on GenBank with between 94% and 99% similarity.

3.2 TTX Standard

The TTX standard shows that, at concentrations ranging from 0.1 to 50 ppm, the retention time ranges from 6.575 mins to 6.752 mins, with an average of 6.695 mins (4 s.f.). The 100% concentration TTX retention time was slightly later at 7.025 mins (4 s.f.) for the average of the three runs at 100% (Table 4). The three runs however revealed differing peak areas for 100 ppm (951, 292 and 287), which were therefore not considered for the concentration gradient (Figure 9). At an excitation wavelength of 384 nm and an emission wavelength of 505 nm, the calibrated concentrations resulted in an R^2 of >0.999, demonstrating a high accuracy of the calibration curve up to 10 ppm. An example of a TTX peak is shown in Figure 10.

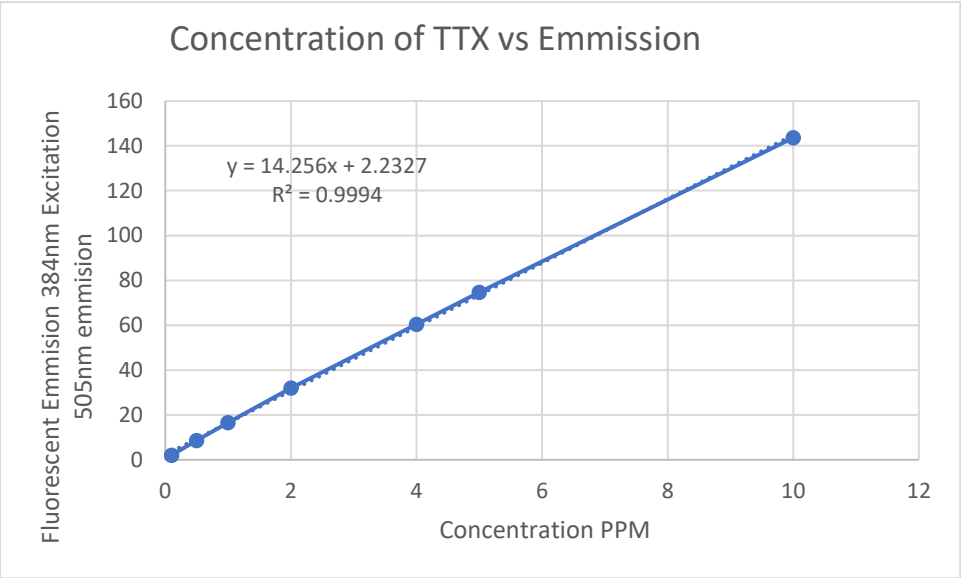


Figure 9. Concentration gradient created from the TTX standard

Table 4. TTX standard prepared using HPLC/UV/Vis/FLD with TTX used immediately and stored at room temperature and using acetonitrile as a solvent

Concentration (PPM)	Time (mins)	Area	Height	Width	Area %	Symmetry
Blank	0.000	0.0	0.00	0.000	0	0.000
Blank	0.000	0.0	0.00	0.000	0	0.000
0.1	6.733	2.1	0.11	0.332	100	0.872
0.1	6.752	2.0	0.10	0.323	100	0.791
0.1	6.752	2.1	0.11	0.327	100	0.828
0.5	6.736	8.3	0.42	0.326	100	0.801
0.5	6.736	8.6	0.43	0.332	100	0.797
0.5	6.724	8.8	0.45	0.328	100	0.855
1	6.713	17.1	0.85	0.336	100	0.805
1	6.727	16.3	0.81	0.335	100	0.809

1	6.710	16.5	0.84	0.328	100	0.828
2	6.714	31.6	1.60	0.334	100	0.794
2	6.695	31.9	1.60	0.337	100	0.787
2	6.693	32.3	1.60	0.338	100	0.769
4	6.689	60.5	3.00	0.318	100	0.750
4	6.689	60.6	3.00	0.321	100	0.758
4	6.681	60.0	2.90	0.319	100	0.752
5	6.681	74.8	3.60	0.323	100	0.739
5	6.673	75.2	3.70	0.320	100	0.750
5	6.657	74.0	3.60	0.323	100	0.742
10	6.668	144.7	6.80	0.333	100	0.717
10	6.658	142.2	6.70	0.329	100	0.738
10	6.663	143.8	6.70	0.334	100	0.723
50	6.575	544.2	17.40	0.432	100	0.391
50	6.721	435.1	15.40	0.397	100	0.489
50	6.629	697.6	20.10	0.491	100	0.487
100	7.059	951.9	25.10	0.633	100	1.860
100	7.007	292.1	13.20	0.331	100	0.676
100	7.010	287.1	13.10	0.329	100	0.680

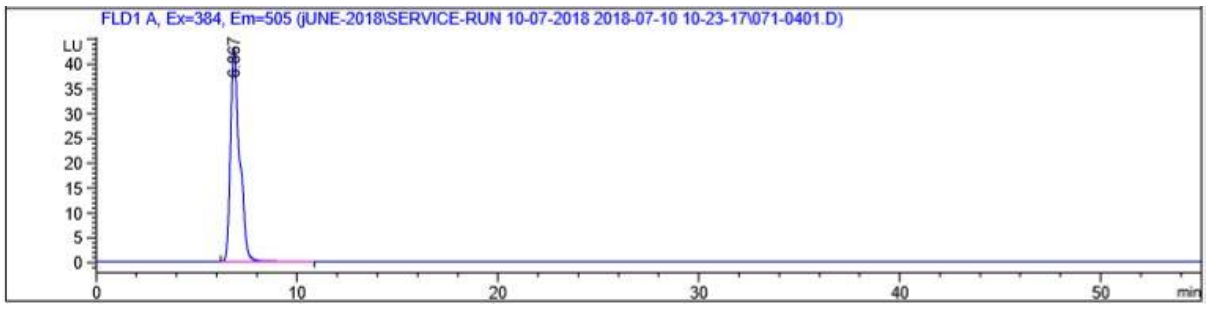


Figure 10. Peak for TTX 100 PPM using HPLC/UV/Vis/FLD with acetonitrile solvent

The results from the additional standard that was run with the TTX that had been stored at room temperature for one month and in acetic acid for one week, showed a retention time that was increased by approximately one minute (Table 5).

Table 5. TTX standard using HPLC/UV/Vis/FLD after TTX was stored for one month at room temperature and one week in acetic acid, using acetonitrile as a solvent.

Concentration	Time	Area	Height	Width	Area%	Symmetry
0.1	7.717	1.2	0.03	0.815	100	0.732

3.3 TTX Detection

The adult roadkill newt and egg samples were run using the newly established method of HPLC/UV/Vis/FLD with the samples dissolved in acetic acid and the results are shown in Table 6 (adult newts) and Table 7 (eggs). Each time indicates a specific molecule that has been detected, which can be compared with the time given for TTX in Table 4. Of the adult newts, three samples revealed the potential presence of TTX as evidenced by peaks with a retention time close to the 6.695 minutes (Table 8). Each sample was run twice, and an average of the peaks that point to TTX was taken. Sample N10 had a peak with an average retention time of 6.540 mins (4 s.f.), sample number N11 had a peak at 6.798 mins (4 s.f., Figure 11) and sample N12 had a peak at 6.790 mins (4 s.f.). Furthermore, samples 5 and 9 also showed peaks which were close to, but not entirely matching the expected retention time of TTX. The results also indicate that other molecules were detected, which is expected in a sample with various unknown components. With one exception likely due to contamination (control X for a run involving samples N9-N13, Figure 12), the applied negative controls either revealed no peaks, or no peak close to the retention time of TTX (Figure 13, Table 6).

Out of the 13 sites sampled using eggs, three showed evidence for the presence of TTX with a peak close to 6.695 mins (Table 7). These were the eggs collected from the site in Wales and, eggs from two sites sampled in France. The Welsh site average peak retention time was 6.962 mins (4 s.f.) and the French sites were an average of 7.037 mins (4 s.f.) for sample 162 and 6.654 mins for one of the runs for sample 205. This peak was, however, not repeatable in the second run of this particular sample. None of the negative controls showed any peaks (Table 7).

Table 6. Results of newt samples run using acetic acid on HPLC/UV/Vis/FLD providing retention times for comparison with TTX retention time, including area, height, width and symmetry, of each peak or substance within the sample

Sample Number	Peak Number	Time	Area	Height	Width	Area %	Symmetry
N1	1	10.78	40.8	1.2	0.5644	2.104	1.843
	2	12.532	1408.7	36.7	0.5833	72.649	1.172
	3	13.874	366	9.2	0.6188	18.876	0.738
	4	15.861	123.5	3.7	0.5279	6.37	0.813
N1	1	10.897	46	1.1	0.7121	3.211	2.586
	2	12.232	1012.2	25.7	0.656	70.681	1.087
	3	13.878	278.9	5.9	0.7273	19.474	0.77
	4	15.887	95	2.8	0.5258	6.634	0.813
N2	1	10.654	25.3	0.68	0.6237	1.529	1.243
	2	12.472	1015.2	28.2	0.5405	61.443	0.961
	3	13.82	464	11.9	0.6077	28.081	0.777
	4	15.878	147.8	4.4	0.5294	8.947	0.816
N2	1	10.578	22.4	0.67	0.559	0.999	1.226
	2	11.814	61.5	2.4	0.4238	2.744	2.019
	3	12.786	1460.9	44.5	0.5256	65.222	1.026
	4	13.877	505.4	14.7	0.545	22.564	0.725
	5	15.866	189.7	5.6	0.5328	8.471	0.823
N3	1	10.594	39.3	1	0.6341	1.761	1.008
	2	11.845	68.1	2.6	0.4283	3.054	1.775
	3	12.814	1895.5	59.2	0.512	85.049	1.006
	4	13.874	225.9	6.9	0.5265	10.135	0.654
N3	1	10.647	40.8	0.99	0.6843	2.318	1.466
	2	11.95	70.1	2.6	0.4245	3.984	4.603

	3	12.695	1403.2	43	0.5258	79.731	0.908
	4	13.876	245.8	7.1	0.5515	13.967	0.73
N4	1	10.968	12.6	0.26	0.8054	1.221	8.763
	2	12.071	911.5	23.4	0.6503	88.193	0.829
	3	13.956	109.4	2.2	0.7475	10.586	0.76
N4	1	10.958	13.6	0.29	0.7668	1.756	3.934
	2	12.059	649.3	16.7	0.647	84.099	0.838
	3	14.126	73.4	1.7	0.7099	9.501	0.591
	4	15.86	35.9	0.98	0.6125	4.644	0.897
N5	1	7.715	2.9	0.14	0.3524	0.696	0.488
	2	10.67	6.4	0.2	0.5308	1.559	0
	3	12.07	335.8	8.9	0.6273	81.619	0.856
	4	14.155	45.9	1	0.7622	11.158	0.545
	5	15.876	20.4	0.64	0.5295	4.968	0.81
N5	1	7.66	14	0.9	0.2584	3.426	0.869
	2	11.196	2.5	0.094	0.4529	0.623	0
	3	12.078	330.3	8.7	0.6324	80.716	0.884
	4	14.17	41.4	0.94	0.7339	10.116	0.695
	5	15.876	20.9	0.65	0.5367	5.119	0.799
N8	1	10.86	13.9	0.28	0.8357	1.875	3.242
	2	12.118	622.2	16.2	0.6412	83.826	0.89
	3	13.817	86.4	1.8	0.8174	11.642	0.576
	4	15.891	19.7	0.64	0.5126	2.658	1.048
N8	1	12.36	913.7	22	0.6193	88.787	1.205
	2	13.813	115.4	2.9	0.6194	11.213	0.687
N1 - N8 Negative Control X	1	10.627	18	0.56	0.5402	1.513	1.891
	2	11.889	22.1	0.82	0.4509	1.854	4.728
	3	12.697	771.7	23.9	0.5165	64.803	0.937
	4	13.897	273.2	7.5	0.5655	22.942	0.748
	5	15.869	105.8	3.1	0.5247	8.888	0.814
N1 - N8 Negative Control X	1	10.658	28.6	0.71	0.6703	2.522	1.323
	2	12.481	778.8	18.9	0.6868	68.727	1.207
	3	13.853	237	6.3	0.629	20.917	0.74
	4	15.874	88.8	2.6	0.5281	7.835	0.811
N1 - N8 Negative Control Y	1	10.728	20.2	0.5	0.6703	1.129	2.273
	2	12.391	1207.8	30.7	0.6551	67.402	1.131
	3	13.887	529.5	13.4	0.6115	29.548	0.794

	4	15.879	34.4	1.2	0.4887	1.92	0.937
N1 - N8 Negative Control Y	1	10.647	25.2	0.67	0.6237	1.096	1.09
	2	11.835	49.1	2	0.4073	2.131	1.921
	3	12.754	1573.4	49.6	0.5063	68.305	0.97
	4	13.912	616.8	17.4	0.5562	26.777	0.787
	5	15.898	39	1.4	0.4747	1.692	0.595
N9	1	7.635	11.6	0.72	0.2688	1.263	0.566
	2	11.003	13.1	0.28	0.7886	1.425	5.483
	3	12.098	860.4	19.9	0.7224	93.656	0.701
	4	14.594	16.9	0.4	0.7052	1.844	0.671
	5	15.866	16.7	0.53	0.5248	1.812	0.789
N9	1	7.663	16.4	0.65	0.4178	1.753	0.571
	2	11.036	16.4	0.22	1.2419	1.752	4.497
	3	12.098	855	19.8	0.7181	91.449	0.678
	4	14.553	24.9	0.49	0.855	2.668	0.95
	5	15.891	22.2	0.59	0.6327	2.378	0.71
N10	1	6.822	7	0.26	0.4428	3.568	3.251
	2	7.453	5.1	0.35	0.2445	2.616	1.732
	3	7.963	27.3	1	0.4561	13.899	0.446
	4	10.516	156.7	4.1	0.6016	79.917	0.775
N10	1	3.005	2.5	0.23	0.1802	0.558	0.526
	2	6.258	2.4	0.13	0.3043	0.549	0.482
	3	7.579	4.1	0.16	0.4167	0.925	0.221
	4	10.786	6.6	0.18	0.5971	1.475	0
	5	12.085	407.4	9.5	0.7144	91.751	0.718
	6	14.752	12.2	0.24	0.8539	2.757	0.944
	7	15.898	8.8	0.29	0.5062	1.984	0.823
N11	1	6.802	7.7	0.27	0.4823	1.735	1.794
	2	7.409	3.5	0.24	0.2444	0.792	1.726
	3	7.949	37.2	1.4	0.4405	8.39	0.458
	4	10.617	394.9	10	0.6079	89.084	0.744
N11	1	6.793	7.3	0.23	0.518	1.628	2.204
	2	7.406	5.5	0.29	0.3194	1.244	0.854
	3	7.934	39.5	1.5	0.444	8.869	0.52
	4	10.604	393.1	10	0.6057	88.259	0.709
N12	1	6.79	8.5	0.31	0.4527	3.388	1.583
	2	7.404	2.5	0.14	0.2836	0.986	1.649
	3	7.927	27.6	0.92	0.5002	11.042	0.531

	4	9.291	2.2	0.085	0.4348	0.89	1.405
	5	10.622	209.4	5.6	0.6268	83.695	0.777
N12	1	6.789	7.7	0.27	0.4671	3.938	1.552
	2	7.323	1.7	0.11	0.2651	0.883	1.158
	3	7.906	18.9	0.71	0.4442	9.707	0.558
	4	9.217	1.6	0.057	0.4533	0.803	1.086
	5	10.701	164.6	4.3	0.5866	84.668	0.761
N13	1	12.239	674.59668	12.88925	0.8223		
	2	14.185	420.2641	6.80692	0.9147		
N13	1	12.154	524.0434	9.84946	0.8281		
	2	14.156	287.82181	4.73841	0.9095		
N14	1	12.161	562.04297	9.92516	0.8702		
	2	14.224	214.94743	3.57516	0.8807		
N14	1	11.898	370.78537	6.3742	0.9054		
	2	14.101	137.54967	2.09497	0.9453		
N9-N14 Negative Control X	1	6.781	2.3	0.053	0.7321	100	0.929
N9-N14 Negative Control X	1	6.714	2.6	0.061	0.7121	100	0.67
N9-N14 Negative Control Y	0						
N9-N14 Negative Control Y	0						

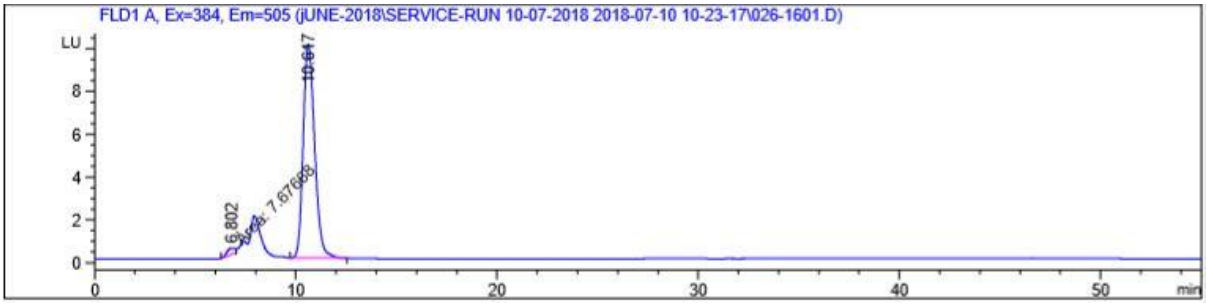


Figure 11. Peaks of samples N11 thought to include TTX, using FLD

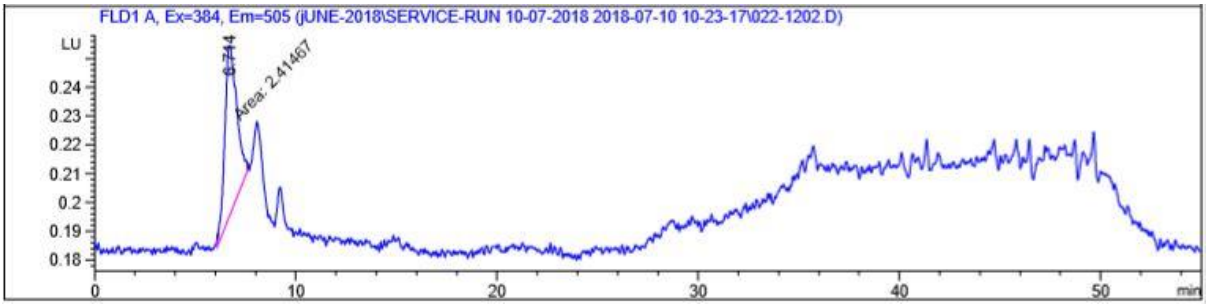


Figure 12. Peaks of negative control X used for samples N9 - N14 including potential TTX as contamination

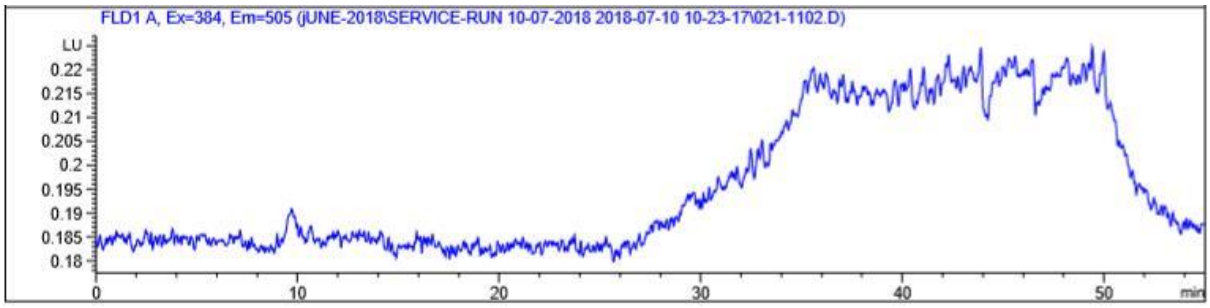


Figure 13. Peaks of negative control Y used for samples N9 - N14 blank as evidenced by lack of peaks

Table 7. Results of egg samples run with acetic acid on fluorometer. For the samples highlighted in yellow see the main text.

Sample Number	Peak Number	Time	Area	Height	Width	Area %	Symmetry
E1	1	10.625	19.2	0.35	0.9191	40.511	0.711
	2	12.708	1.5	0.043	0.5906	3.185	1.038
	3	13.855	18.3	0.52	0.5809	38.623	0.828
	4	32.044	8.4	0.081	1.7177	17.681	0.569
E1	1	10.622	19.4	0.36	0.91	42.494	0.745
	2	12.741	1.9	0.054	0.5867	4.135	1.414
	3	13.867	18.3	0.52	0.5856	40.075	0.84
	4	31.94	6.1	0.073	1.3942	13.296	0.637
E1 Negative Control	0						
E1 Negative Control	0						
E2	1	36.76	52170.1	1107.2	0.634	93.236	4.39
	2	37.693	236.6	10.8	0.3683	0.423	0.734
	3	39.251	1182.8	46.1	0.3986	2.114	2.353

	4	39.817	2365.1	80.2	0.478	4.227	0.499
E2	1	36.567	49358.7	985	0.7121	92.99	1.897
	2	37.684	223.5	10.2	0.3545	0.421	0.733
	3	39.246	1137.6	43.2	0.4175	2.143	2.555
	4	39.807	2359.6	79.6	0.4735	4.445	0.498
E2 Negative Control	0						
E2 Negative Control	0						
E3	1	10.625	3.8	0.084	0.7527	11.999	1.19
	2	11.82	1.4	0.034	0.7018	4.554	0.98
	3	12.643	1.1	0.037	0.4805	3.332	0.596
	4	13.838	25.3	0.7	0.6046	80.115	0.853
E3	1	10.571	4.1	0.088	0.7701	15.013	0.942
	2	12.546	2.6	0.054	0.8056	9.594	1.72
	3	13.788	20.5	0.59	0.5782	75.393	0.833
E3 Negative Control	0						
E3 Negative Control	0						
E4	1	10.598	6.8	0.15	0.7414	21.128	1.035
	2	13.823	25.5	0.71	0.5939	78.872	0.851
E4	1	10.561	10.8	0.2	0.8963	29.581	0.619
	2	12.247	1	0.029	0.5721	2.757	0.512
	3	13.836	24.6	0.7	0.5894	67.661	0.907
E4 Negative Control	0						
E4 Negative Control	0						
E5	1	10.644	7.4	0.14	0.8773	28.69	0.972

	2	12.701	2.3	0.047	0.819	8.991	2.214
	3	13.853	16	0.46	0.5873	62.319	0.849
E5	1	10.641	5.1	0.11	0.7733	25.954	1.023
	2	12.542	1.8	0.027	1.1027	9.069	2.211
	3	13.774	12.8	0.36	0.5827	64.977	0.862
E5 Negative Control	0						
E5 Negative Control	0						
E6	1	10.647	11.9	0.21	0.9255	30.283	1.019
	2	12.724	1.3	0.035	0.6328	3.345	1.498
	3	13.838	26.1	0.73	0.595	66.372	0.885
E6	1	10.635	14.3	0.24	0.9904	34.592	0.937
	2	12.708	2.2	0.048	0.746	5.248	2.104
	3	13.831	24.9	0.7	0.589	60.161	0.868
E6 Negative Control	0						
E6 Negative Control	0						
E7	1	10.843	2.4	0.055	0.7127	32.843	1.841
	2	12.031	1.4	0.027	0.8636	19.478	0.875
	3	13.55	3.4	0.11	0.5329	47.679	0.855
E7	1	10.787	2.4	0.056	0.7167	35.043	1.063
	2	12.023	1.2	0.027	0.7273	17.451	0.79
	3	13.55	3.2	0.098	0.5521	47.505	0.947
E7 Negative Control	0						
E7 Negative Control	0						
E8	1	10.669	5.2	0.096	0.9099	30.286	0.842
	2	12.632	1.6	0.035	0.7519	9.245	1.581
	3	13.81	10.5	0.29	0.6039	60.469	0.882
E8	1	10.686	5.2	0.096	0.9057	29.913	0.946

	2	12.63	1.9	0.042	0.7363	10.754	1.584
	3	13.831	10.4	0.28	0.6059	59.333	0.879
E8 Negative Control	0						
E8 Negative Control	0						
E9	1	10.679	3.5	0.072	0.8183	19.688	1.164
	2	11.858	1.5	0.037	0.6529	8.141	0.62
	3	13.788	13	0.36	0.6028	72.171	0.976
E9	1	10.704	2.7	0.061	0.549	15.932	1.964
	2	11.837	1.3	0.036	0.6036	7.498	0.913
	3	13.774	13.2	0.36	0.6125	76.57	0.899
E9 Negative Control	0						
E9 Negative Control	0						
E10	1	6.959	1.1	0.022	0.8287	45.964	0.639
	2	8.12	1.3	0.042	0.5068	54.036	0.477
E10	1	6.965	1.1	0.021	0.8741	46.547	0.605
	2	8.142	1.3	0.041	0.5245	53.453	1.033
E10 Negative Control	0						
E10 Negative Control	0						
E11	1	6.654	1.9	0.11	0.2984	21.704	2.761
	2	7.055	4.5	0.17	0.4538	51.51	1.333
	3	8.59	1.2	0.05	0.3868	13.266	0.99
	4	9.763	1.2	0.057	0.35	13.52	0.473
E11	1	8.044	7.7	0.28	0.4586	15.055	1.01
	2	8.42	4.4	0.22	0.3051	8.539	0.254
	3	9.958	2.5	0.12	0.3461	4.967	1.68
E11 Negative Control	0						

E11 Negative Control	0						
E12	1	10.776	2.4	0.049	0.604	6.848	1.328
	2	12.766	9.2	0.27	0.5674	26.586	1.394
	3	14.357	20.7	0.33	1.0481	59.878	1.463
	4	15.819	2.3	0.073	0.5286	6.688	0.725
E12	1	11.032	1.2	0.022	0.6518	8.1	0
	2	12.279	4.7	0.12	0.6715	31.589	1.411
	3	13.666	2	0.059	0.4294	13.134	2.376
	4	14.458	5.7	0.16	0.5964	38.063	0.591
	5	15.883	1.4	0.044	0.5138	9.114	1.005
E12 Negative Control	0						
E12 Negative Control	0						
E13	1	7.01	287.1	13.1	0.3293	100	0.68
E13	1	7.063	7.1	0.16	0.7238	47.432	1.651
	2	9.094	1.5	0.049	0.4954	9.702	0.6
	3	9.583	1.1	0.071	0.2597	7.411	0
	4	10.102	4.3	0.088	0.8181	28.777	1.501
	5	11.714	1	0.038	0.4386	6.678	0.547
E13 Negative Control	0						
E13 Negative Control	0						

3.4 LC-MS analysis

Each of these three runs using LC-MS took 3 mins, with the peaks typically eluting from approximately 0.2 mins. Figures 14-16 show the peaks representing specific molecular masses that were revealed at this time. The molecular mass (M) of TTX is 319.1016, ionising

with hydrogen (1.0078) to give an $M+H^+$ ion of 320.1094. The observed peaks did not coincide with peaks expected for TTX. The results from the LC-MS analysis at Manchester Metropolitan University showed that TTX was unlikely to be present in each of the three newt samples. If a TTX peak had been found, to prove this was not a substance that was found in the acetic acid, it could be compared with the acetic acid blank which was run first. No such peak was seen in either the blank or the samples, indicating that in a previous experiment (results shown in Table 6) negative control samples N9-N14 that had shown up a similar peak to TTX were likely erroneous and that the acetic acid used does not contain a substance with a similar mass that can be mistaken for TTX, either naturally or by contamination. An unknown chemical compound may have previously reacted with the fluorescent marker in the HPLC/UV/Vis/FLD experiment, which could have eluted at a similar time to that which was expected for TTX, explaining the ambiguous result.

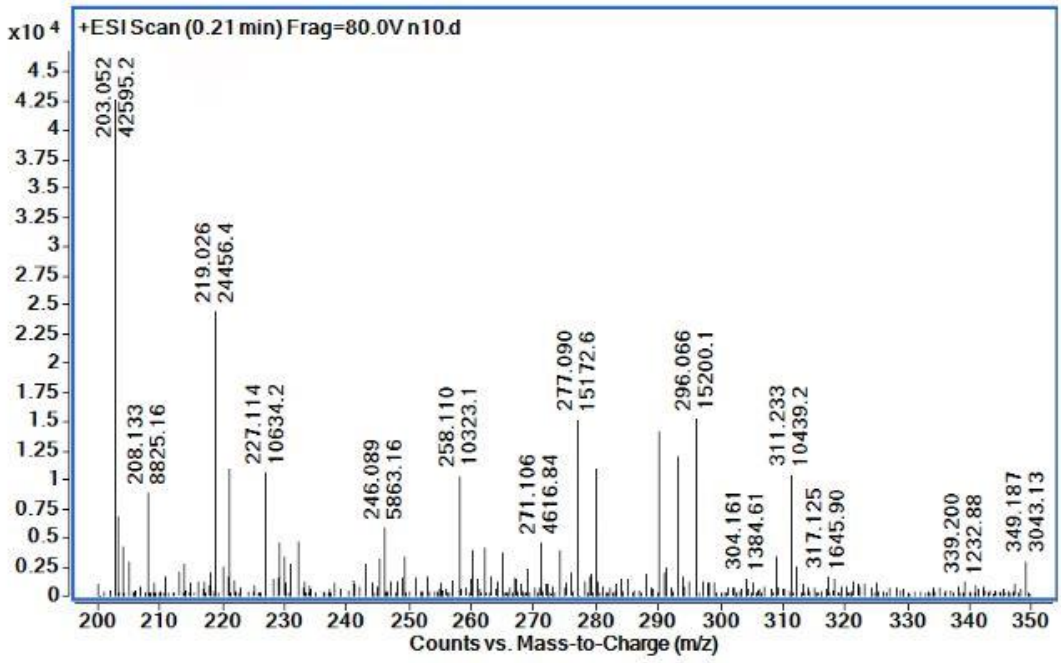


Figure 14. Screenshot of peaks seen at 0.21 mins of newt sample N10 run on LC-MS with each peak indicating mass of substance eluted at this time, showing no peaks at 320.1094 the expected mass for TTX

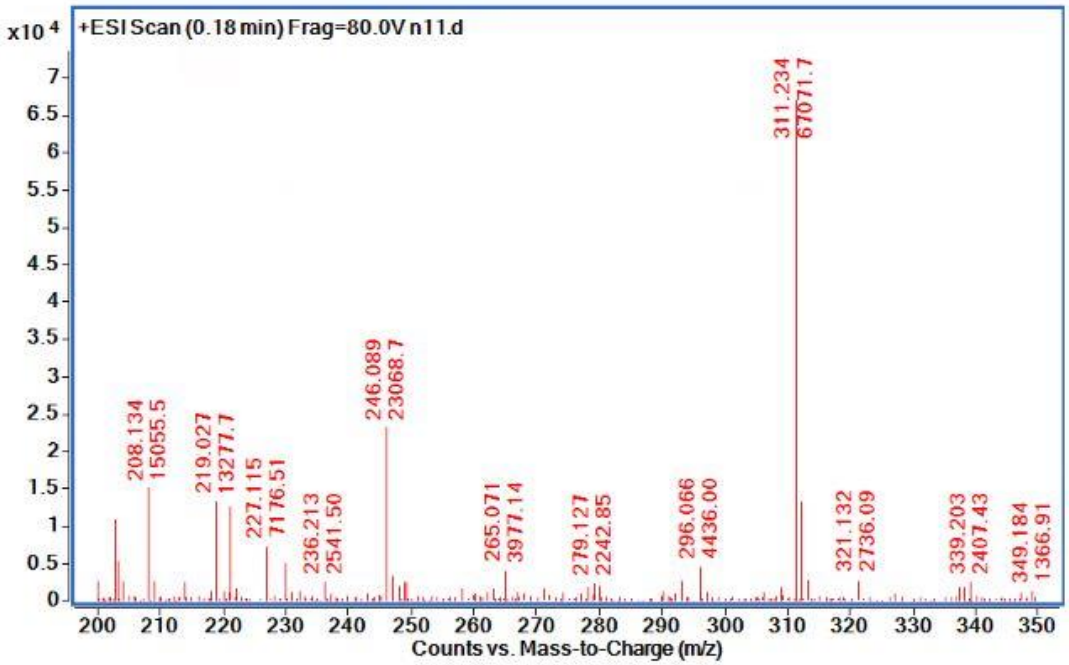


Figure 15. Screenshot of peaks seen at 0.18 mins of newt sample N11 run on LC-MS with each peak indicating mass of substance eluted at this time, showing no peaks at 320.1094 the expected mass for TTX

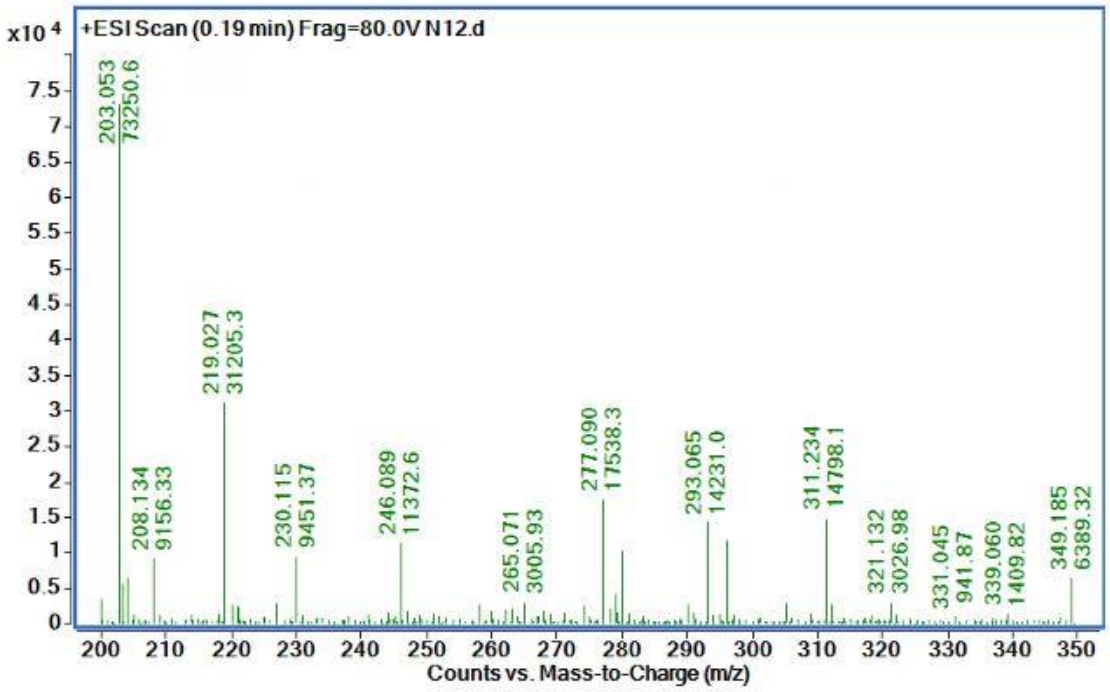


Figure 16. Screenshot of peaks seen at 0.19 mins of newt sample N12 run on LC-MS with each peak indicating mass of substance eluted at this time, showing no peaks at 320.1094 the expected mass for TTX

4. Discussion

The results of this study ultimately indicated that TTX is highly unlikely to be present in any of the investigated samples. Because of this, some of the aims of the study which sought to compare the levels of TTX in different geographical locations, as well as comparing TTX levels with the associated predations levels upon each population, were not achieved. It was also not possible to determine a difference between the levels of TTX in different parts of the newt to each other and that of eggs, as no positive results were obtained. The results of the thesis , however, revealed new methodological insights, including sample preparation and TTX detection, which can be used for future studies. Genetic analysis was also performed and some species were able to be identified.

4.1 Fieldwork and Study Sites

In total, there were five locations used for fieldwork in this study; one each in Wales, Scotland and France and two in England. One of the locations in England was used for collection of roadkill newts, and all other locations were used for the collection of eggs. These locations were chosen due to the known abundance of small bodied newts in these areas, to which collection of eggs would have little ecological impact. Studies show that geology, soil and water conditions have an effect on the species of newt that populate given areas, whereby *L. helveticus* appears more tolerant to acidic conditions than *L. vulgaris* (e.g. Griffiths 1996). Griffiths (1993) showed that under acidic conditions, snapping behaviour of *L. vulgaris* and *L. helveticus* was reduced, whereas feeding behaviour of *T. cristatus* was unaffected. On the other hand, Griffiths and de Wijer (1994) showed that *L. vulgaris* and *L. helveticus* are more tolerant of acidic conditions than *T. cristatus*, with an 80% hatch rate for *L. vulgaris* and *L. helveticus* compared to zero for *T. cristatus*. Brady and Griffiths (1995) later

showed that while growth was inhibited in both *L. vulgaris* and *L. helveticus* under acidic conditions, snapping behaviour was reduced in *L. vulgaris* compared to *L. helveticus*, making *L. helveticus* the more tolerant to acidic conditions.

The roadkill adult newts were collected from Lumbutts Road, Todmorden on the border between West Yorkshire and Lancashire in the north of England. The stretch of road they were collected from was surrounded by hilly, open moorland and agricultural land (Figures 17 and 18). This is ideal habitat for newts as well as their predators and it was hypothesised that some level of TTX would be beneficial to the newts here. However, as TTX was not detected and the newts are seen in abundance in this location, it could be suggested that TTX is not a necessary predator deterrent in this case. On the other hand, the lack of TTX detected does not guarantee the absence of the toxin in all individuals.

As newts were road kill they had varying degrees of freshness, depending on whether they had been killed on the night of collection or from a previous night. It was assumed that none were more than 24 hours old; from personal experience of patrolling this area, newts are usually taken by predators within a day or being killed. Freshness of the newt may have affected the concentrations of TTX present in the newt by the time it was processed for sampling (see also below). Both *L. vulgaris* and *L. helveticus* were seen during fieldwork at this location.

Figure 17. Landscape surrounding Lumbutts Rd, Todmorden, England

Figure 18. Agricultural landscape surrounding Lumbutts Rd, Todmorden, England

The additional site in England was Egerton Quarry in Bolton in the northwest of England, used for collecting eggs. This site is a disused quarry which has given way for woodland with bodies of water of various sizes. The site consists of multiple ponds suitable for newt habitat with no barriers to prevent migration between ponds. The site is home to various aquatic invertebrates which may predate upon the newts or newt eggs. Because of this, detecting TTX would suggest a specific predator avoidance role in any of the newt populations here. The only small-bodied newt so far recorded at Egerton quarry is *L. helveticus* (David Orchard, Amphibian and Reptile Group South Lancashire, 2019, personal communication to R. Jehle).

The samples of eggs obtained from Scotland were from Inverness, located on the east coast of the northern region, and included locations from farm land, as well as three Sustainable Urban Drainage Systems (SUDS). These SUDS are artificial ponds designed to collect surface water run-off from urban environments. The initial tests on the eggs from Scotland (samples E2 – E9 in Figure 9) using HPLC/UV/Vis/MS/FLD methods did not provide any peaks that could be said to be TTX. These egg samples were not tested on the more sensitive LC/MS equipment, therefore with the results that were obtained for these samples, a reliable

result to determine a difference between levels of TTX in newts in urban and rural ponds was not possible. This would be an interesting point for future study as the predation upon each habitat would differ and therefore it may be hypothesised that TTX levels may differ. The species of these eggs were assumed to be *L. helveticus* due to no known sightings of *L. vulgaris* in this area (R. Jehle, 2019, personal communication, 1st October; see also Miro et al. (2017), Rae et al. (2019). The species identification in this case was not necessary as no TTX was detected.

The final British field work site in Wales was located at Morfa Dyffryn, which is on the west coast. This sample (E10) presented an ambiguous result with a peak that was close to that of the TTX standard, shown in Figure 9. However, it was assumed that as the further analysis of three other samples with a similar peak proved to not contain TTX, that this sample also did not contain TTX. The similar peak was assumed to be coincidence and unrelated to TTX. Eggs were collected from a ditch network and species was identified as *I. alpestris*, a species not native to Britain and classified as invasive. Morfa Dyffryn is a coastal area consisting of dune systems with bare sand areas, as well as salt marsh and grassland (Natural Resources Wales, 2020). *I. alpestris* are known to have populated this area from sightings and from the collection of eggs for this study. According to Natural Resources Wales (2020), *T. cristatus* are also found here. The habitat is suitable for *I. alpestris*, *T. cristatus*, and *L. helveticus*. Likely introduced*, L. vulgaris* were also recorded (R. Jehle, 2020, personal communication, 31st August).

Eggs were collected from cattle ponds Mayenne in northwest France, near to the village of Jublains. This area is a well surveyed area for newt species and the surrounding habitat is described in Jehle & Arntzen, (2000) and Jehle, Bouma, Sztatecsny & Arntzen (2000), as

hedgerows, pasture and orchards. Jehle et al. (2000) also show in their study three species of newt residing in the sampled study ponds: *T. cristatus, T. marmoratus* as well as *L. helveticus*. As *T. cristatus* and *T. marmoratus* are large bodied newts, their eggs were distinguishable from the small bodied *L. helveticus* eggs that were selected for this study. Despite showing different resource use, these newts have overlapping habitat (Jehle et al., 2000). Should one species be found to contain TTX, it would be interesting to compare the levels of toxin in these individuals, to individuals of different species from the same area.

As TTX has been found across newts in European countries, including examples in *L. vulgaris* and *I. alpestris* in Germany (Yotsu-Yamashita et al., 2007), a study based on north-western European sites also has the potential to shed further light into whether biogeography, and in particular post-glacial recolonisation, can be linked to the local ability of populations to produce TTX. Whilst this study does not necessarily provide any positive results to add to the current knowledge of differing levels of TTX over different geographical locations, other studies have provided more information. During glacial periods, many European species survive in glacial refugia traditionally assumed to be located in southern European regions, leading to a pattern of decreasing genetic diversity towards more peripheral, northern localities (Chung et al., 2018). While no detailed large-scale studies exist on small-bodied newts, *T. cristatus* have been shown to be less genetically diverse in north-western European populations, both with respect to neutral genetic variation as well as adaptive variation potentially linked to disease resistance and immune response (Babik et al., 2009). If TTX is synthesised by the newt itself, less genetically diverse populations (i.e. more northerly populations) may therefore have lost this ability. However, the recent evidence that TTX production is strongly linked to the skin microbiome (Vaelli et al., 2020) further suggests that genetic variation and TTX presence might not be directly linked and therefore,

genetic diversity may not be an important factor to determine the presence of TTX in local populations.

4.2 Storage and Transportation

Both egg and whole newt samples may degrade either in transit to storage at the laboratory, or during preparation for analysis, potentially affecting TTX detection. The solution in which samples were stored may have had an effect on the form of TTX in the sample. As acetonitrile was the first choice of solvent, the French eggs were stored in acetonitrile. Eggs from Scotland were stored in the pond water they were collected from and were then refrigerated for several days before being frozen. Some eggs had hatched by the time they were frozen, and one pond sample had to be disregarded entirely due to all of the eggs being hatched already. Eggs from Egerton Quarry were stored in pond water, immediately cooled with an ice pack and frozen within half an hour. Welsh egg samples were collected in the pond water and were frozen within a few hours. It is not possible to assess to what extent differences in storage may have affected the results. Due to changes in the protocol (from acetonitrile to acetic acid) and distance of each location from the laboratory, the samples could not be treated in the same way and some samples were frozen later after being collected than others. TTX is heat stable and for example, is still present in puffer fish even when cooked (Bane et al., 2014). Therefore, it is unlikely that the fact that they spent in the order of two days before being frozen, had much influence on the levels of TTX that may have been present. However, particularly the effect of storage in alternative solvent on the findings of the present study are unknown.

4.3 Sample Preparation, LC-MS and Repeat Experiments

Aside of differences between species and populations, this study also aimed to investigate whether different body parts of newt would prove more appropriate for sampling, due to ease of dissection or higher concentrations of TTX. Therefore, the initial experiments included preparing various samples with ventral, dorsal, tail, and limb skin samples. Due to the small size of the newt and the difficulty of dissection with the available equipment, limb and tail sections included muscle and bone. As TTX was not detected, it is not possible to say whether either part of the newt is this case was more appropriate. Previous studies showed that dorsal skin was the most likely to contain TTX (Hanifin et al., 2004). This section has thus far proven to be an adequate section of tissue to remove from the newt and prepare for sampling, therefore, this was successfully used for future experiments.

Due to availability of consumables, acetonitrile was chosen as the initial solvent in the sample preparation. The acetonitrile did not provide any usable or meaningful data, thus indicating that this should not be used as a solvent in this case. After further consultation of the literature (Yotsu-Yamashita, Toennes & Mebs, 2017; Johnson et al., 2018), acetic acid was chosen as a new solvent. The decision to use the acetonitrile and dorsal skin tissue established the successful sample preparation protocol that was used for the forthcoming experiments. An additional note regarding the sample preparation protocol was established when the samples that were prepared for LC-MS analysis were left in the fridge for approximately one month. It was discovered that these samples had turned cloudy, which is not suitable for chromatography. When the samples were stored in the freezer and only removed an hour before analysis, they remained clear. It is therefore important to add to

the established protocol that samples are run shortly after preparation or stored in a freezer until analysis.

This study aimed to determine if the methods of HPLC/UV/Vis, FLD and LC-MS are successful at detecting TTX in European small bodied newts with the applied methods of preparation. While each of the methods did detect various molecules in the samples, including possible candidate TTX peaks during the HPLC/UV/Vis experiments, they did not unambiguously detect TTX with certainty. This is more likely due to the absence of TTX rather than false negative results especially in the case of the HPLC/UV/Vis experiments, as evidenced by contamination in negative controls. In addition, the LC-MS experiments did not detect TTX, which, as more sensitive equipment, adds further evidence to the likelihood of no TTX present in the samples. In regards to the LC-MS however, generic LC-MS parameters were used. If these parameters had been optimised for TTX, this would have increased the sensitivity to measure sub PPB (parts per billion) levels. Given that low amounts of TTX were predicted, this would be beneficial for these samples and should be used in future research. The LC-MS was in this case not optimised for TTX as to do this would require further TTX sample with a known concentration, which would then be prepared with the acetic acid following the same protocol as the sample preparation. This consumable was no longer available due to time and financial constraints. Thus, it is possible that TTX was present in the sample yet remained undetected due to the low concentration.

These methods have been used successfully to detect TTX in various studies (Asakawa, Ito & Kajihara, 2013; Chen et al., 2011; Yan, Yu & Li, 2005), including trace amounts of TTX. For further experiments to determine analogues and for species that have already been shown to contain TTX in their respective countries, liquid chromatography with tandem mass

spectrometry is recommended by EFSA Panel on Contaminants in the Food Chain (CONTAM, 2017). Chromatography is also used in conjunction with UV/Vis or FLD methods of detection. Worsfold (2019) states that UV/Vis is not commonly used for identifying analytes, and therefore would likely not be useful for samples which are being analysed for the presence or lack of TTX. It is, however, useful for measuring concentrations. UV/Vis may be useful when TTX is known to be present, such as in a puffer fish sample in which TTX is commonly found, but levels of TTX are in question. As an alternative, FLD, which targets fluorescing molecules, can be used for identifying as well as quantifying TTX. For detecting and quantifying TTX in European newts, FLD used with HPLC methods would be an ideal method (for its use to detect TTX in various species see Asakawa et al., 2013; Yotsu-Yamashita et al., 2017). Cell bioassays and immunoassays cannot distinguish between TTX and its analogues (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2017). As any form of TTX has yet to be discovered in north-western European newts, this approach would still be useful to apply.

Due to the assumed low levels of TTX in these samples, and ambiguous results thus far, the samples were run on a more sensitive LC-MS machine using HR-MS TOF at Manchester Metropolitan University. The results proved reliable and precise, which is consistent with many other studies that have used this method to detect TTX, even at trace amounts (Shalabai et al., 2016). Due to financial constraints, only three samples were run. This method would have been valuable for use on all the samples that were collected, including all the eggs if this experiment was to be repeated. While LC-MS detected various molecules in the samples, none of these could unambiguously be shown to be TTX. As with the previous methods used in this study, this is likely due to the absence of TTX rather than false negative results. It is possible that the storage time had an effect on the results of this

experiment, as the samples had been stored, albeit in a freezer, for approximately 18 months before they were prepared.

Ideally, no newts should be harmed in attempts to detect TTX. Indeed, European authorities forbid the use of live animals when an equivalent method is available (Reverté, Soliño, Carnicer, Diogène & Campàs, 2014). Some studies have successfully used skin punches of 2 mm in diameter for TTX detection in newts (Bucciarelli et al., 2014). This was, however, in the California newt *T. granulosa* which is larger than the species used in this study, for which taking 2 mm skin punches would likely seriously affect their health. Furthermore, *T. granulosa* usually has much higher levels of TTX in its skin (up to 3000 μg/g) compared to the 8 μg/g found in central European *L. vulgaris* and *L. helveticus* (see Table 1, and references therein). Therefore, while a smaller sample size may work for *T. granulosa*, it may not be enough to detect the low amounts expected in *L. vulgaris* and *L. helveticus*. As an alternative, eggs have been shown to contain TTX (Hanifin, Brodie III & Brodie Jr, 2003; Gall, Stokes, French, Brodie III & Brodie Jr, 2012) and this is a much more ethical option for testing. It does however mean that sample collection would be limited to during breeding season.

4.4 Species Identification

A further aim of the study was to identify the species that had been collected, so that if TTX were to be found, the concentrations could be compared to other studies which had used the same species. The adult newts collected were initially given a speculative species identification based on their appearance. All newts were confidently categorised as limited to one of either *L. vulgaris* or *L. helveticus*. Despite some reasonable differences in *L.*

vulgaris and *L. helveticus*, many of the newts were apparently female and therefore more difficult to distinguish. As *L. vulgaris* and *L. helveticus* largely share similar habitats, predators, and prey, this was not crucial. Nevertheless, should TTX be found, the identification of the species is important for further research regarding the occurrence of TTX through populations and around the country. It would also be interesting to examine why one species possessed TTX, and another very similar species did not, if this were the case.

The eggs were identified based on previous research and the likelihood of the species residing in the area from which they were taken. Egerton quarry is known to only contain *L. helveticus* (R. Jehle, 2019, personal communication, 1st October) and so eggs collected from here were categorised with reasonable confidence as *L. helveticus*. It is, however, possible that *L. vulgaris* have migrated into the area since this. It is also interesting to note that great crested newt eggs were sighted during field work in this area. Eggs from Wales were categorised as *I. alpestris*. Adult *I. alpestris* were sighted residing in this area, furthermore, the eggs of this species are slightly darker and larger in appearance. Because of this, these eggs were confidently categorised. In France and Scotland, *L. helveticu*s are the only species known to reside in the sampling locations. This is again not as reliable as genetic analysis, however, as all the eggs were originally expected to be used in the sampling process, genetic analysis was not performed on the eggs. Eggs are relatively small, laid individually, and often both species share a pond. Therefore, it may be difficult to perform genetic analysis to determine the species identification of each egg.

To determine exact species classification, genetic analysis was performed on the adult newts. The results of this proved to be somewhat inconclusive for some tested individuals.

Whilst four individuals were identified as *L. helveticu*s, others were either not identified as any species, or were identified as bacteria, indicative of sample contamination, or due to the age of the roadkill found (carcasses will be colonised by microorganisms after the death of individuals). If this study was to be repeated, care should be taken to avoid contamination and to perform the analysis with precision and with fresh reagents and samples.

4.5 Impacts of Tetrodotoxin

The production of tetrodotoxin is hypothesised to be an anti-predator mechanism (Williams et al., 2010; Gall et al., 2011). Recently, an additional hypothesis has been proposed which suggests that TTX may have evolved as a protection against parasites and infections (Johnson et al., 2018). After finding a mis-match between TTX levels in newts, compared with levels of resistance in their garter snake predator, Johnson et al. (2018) analysed *T. granulosa* and *T. torosa* for levels of TTX compared with parasitic infection. They found that the levels of TTX negatively correlated with parasite richness, and interestingly, the likelihood of contracting chytrid fugus and ranavirus. Despite this data, they point out that this correlation does not imply causation. The newts with higher levels of TTX could simply be healthier in general, and therefore more resistant to the parasites for other reasons. Additionally, the resources that allow for the uptake of TTX, or that stimulate TTX production, may independently affect immune function. As well as potential parasite protection, TTX is used as a venom for capturing and immobilising prey in the blue ringed octopus and a polyclad flatworm (Sheumack, Howden, Spence & Quinn, 1978; Ritson-

Williams, Yotsu-Yamashita & Paul, 2006). Tetrodotoxin therefore has potentially various functions across the wide range of species it is found in.

The levels of TTX that were hypothesised to be detected in this study were low, and unlikely to have any significant effect on the predators of newts. The results indicating that TTX was unlikely to be present is reasonable, considering the predators that these newts face. Predators of newts include birds, fish and small mammals such as foxes, badgers and hedgehogs (e.g. Griffiths 1996). With the number of predators that *L. vulgaris* and *L. helveticus* face, they continue to thrive in Britain and France. A population with greater predation threats may benefit from higher levels of TTX and it would be interesting to conduct a comparison between these populations and populations that have relatively low predation, to determine to what extent predation influences TTX levels. Regardless of whether the toxin has an endogenous or exogenous origin in newts, it has a cost to produce or uptake (Hanifin, 2010). Hanifin (2010) also suggests that there is a cost to the newt in becoming resistant to TTX, a trait that is needed in order to possess the toxin. Because of this, it must be beneficial for survival and fitness. Many of the risks that newts face are from external influences to which a toxin would have no effect, such as vehicles and man-made structures obstructing migrations paths, natural disasters and extreme weather. Possession of this toxin therefore may not be the best use of energy, particularly in more urban populations. Had TTX been found, the concentrations of TTX in the newt eggs from urban ponds of Scotland could have been compared to that of the rural ponds to test this hypothesis. The results, nevertheless, do not rule out the possibility of newt populations from different regions, or indeed different individuals from the same regions, possessing TTX. It would be interesting to conduct further research into the levels of TTX on populations facing differing levels of predation in Europe. Research on the newt/garter

snake relationship suggests that predation does have an effect on TTX levels (Vaelli et al., 2020). In species that possess high levels of TTX, the toxin is an efficient anti-predator device. Therefore, these British and French newts have the potential to evolve higher levels of TTX, which would in turn be more beneficial as a defence mechanism. In the future, this may lead to a similar arms race as the garter snake/newt relationship, whereby predators of British newts develop and evolve a resistance to the toxin. Previous research has shown that different populations of newts have varying levels of TTX (Lorentz et al., 2016). These levels in turn affect whether newts choose to disperse or remain at their natal populations, and whether they are preyed upon. Garter snakes are known to be able to detect levels of TTX in newts and if levels are too high, they will reject the prey (Williams et al., 2010). It may be worthwhile to conduct additional research to classify the newts into genetically distinct populations. The adult newts collected in this study came from Todmorden in West Yorkshire, however, even the newts collected from the same stretch of road may have been breeding in different ponds and thus still may have differing levels of TTX.

Despite no TTX being detected in this study, the colouration of the newts may suggest otherwise. Pires et al. (2005) observed that anurans with TTX also displayed aposematic colouration, whereas anurans with no TTX were cryptic in colour. Therefore, they suggest that colouration may be a warning to predators to advertise the toxin. The same may be said for other terrestrial species that display aposematic colouration and possess TTX. Newts with brightly coloured ventral surfaces display defensive poses which present this colourful underside to predators (Kupfer & Teunis, 2001). This suggests that colour affects predator-prey relationships. Many species of newts, *L. vulgaris* and palmate included, display mostly cryptic dorsal colours which help them to camouflage in their habitats. Figure 19 shows one of the newts used in this study. Whilst not as bright as the well-known newt species that

possess TTX, such as *T. torosa*, this individual does still show a definite difference in ventral colouration compared to the dorsal surface of the skin. This may suggest that *Lissotriton* historically possessed toxins, TTX or otherwise or that it currently possesses a toxin that was not detected in this study. Alternatively, it may be that this colouration evolved as a Batesian mimic, of species that are poisonous and display aposematic colouration. *Icthyosaura alpestris* also possesses a brighter ventral skin colour than *L. helveticus* and *L. vulgaris*, which may suggest it is more toxic. However, this species still did not show any levels of TTX as found in this study and so there is no evidence to suggest the connection between TTX and colouration in this case.

Figure 19. Underside of newt used in this study, captured as road kill in Todmorden, West Yorkshire (2018) speculative identification female L. helveticus

The origin of TTX in newts is now thought to be bacterial. Vaelli et al. (2020) succeeded in growing the bacteria they had collected from specimens of *T. granulosa*. These bacteria

were then tested for TTX using LC-MS, and four genera (*Aeromonas*, *Pseudomonas*, *Shewanella* and *Sphingopyxis*) contained TTX. Cardall et al. (2004) showed that upon being subject to electric stimulation that forced the newts to secrete up to 90% their toxin, newts were able to regenerate TTX levels after 9 months despite being denied access to TTX containing diets. Vaelli et al. (2020) suggest this provides evidence that newts do not derive TTX via the food chain, but does not rule out symbiotic origins for TTX in newts. Vaelli et al. (2020) also offer criticism of the widely cited study by Lehman et al. (2004) in which a bacterial origin of TTX in newts is thought to be impossible due to the lack of amplification of bacterial DNA, by suggesting that the sequencing approaches used were limited at the time of the study. *Sphingopyxis* are a bacteria that have not previously been associated with TTX, and Vaelli et al. (2010) suggest that it may be unique to freshwater species. There is potential for other species to evolve resistance and uptake of these bacteria.

In addition to this discovery of a previously unknown bacteria containing TTX, many new marine species have recently been found to possess TTX. These species have also been shown to be moving into new waters. Most of the cases of TTX poisoning happen in Japan, although cases have been reported in Malaysia, New Zealand, USA, and the eastern Mediterranean (Cohen et al., 2009; Bentur et al., 2008; McNabb et al.,2010; Suleiman, Muhammad, Jelip, William & Chua, 2017). Reunion Island in the Indian Ocean had its first reported TTX poisoning for 24 years in 2013 (Puech et al., 2014). Puech et al. (2014) discuss the importance of increased awareness also for the Red Sea through to the Mediterranean Sea due to the Lessepsian Migration of species via the Suez Canal. This man-made structure allows the fish to move through to populate a new area. Species that contain TTX have been caught in the Mediterranean Sea, and have resulted in TTX poisoning. A case study review by Bentur et al. (2008) revealed thirteen cases of TTX poisoning in the eastern

Mediterranean between 2005 and 2008. They attribute these cases to the Lessepsian Migration and discuss the need for increased awareness of TTX in these newly colonised areas. More recently, TTX has been found in bivalves and gastropods in Dutch and South England coastal waters (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2017; Katikou & Vlamis, 2017).

Pufferfish, the most famous species associated with TTX, are integrated into pop culture and are not usually shown in a dangerous light, appearing as friendly characters in children's movies (Finding Nemo, 2003). As well as this, TTX has been misrepresented in movies (Captain America: The Winter Soldier, 2014). This misrepresentation furthers the ignorance surrounding pufferfish and TTX. Folklore has also contributed to the lack of respect for this deadly toxin, by presenting an apparent cure of pickled cabbage. There is no evidence that this has any effect on TTX poisoning, yet this presents a supposed safety net for consumers of pufferfish or other TTX bearing species. Despite the number of TTX poisoning cases, puffer fish are a part of the Japanese culture and will likely continue to be consumed. Health warnings from scientists about the potential dangers of the other living organisms we share the planet with, often have little impact on the choices we humans make, with the current Covid19 pandemic as a prime example (New Scientist, 2020).

Newts are not usually eaten and so the toxicity of newts does not pose a great threat to human life. However, there is still some issues which cause concern. Newts are often kept as pets, being readily available in pet shops, or from online stores selling exotic animals or alternatively collected from the wild. Often, buyers are not aware of the species they are buying or the dangers they may pose. In the USA, a two-year-old child bit the tail off of a *T. granulosa* that had been kept as a pet and which are known to contain TTX. Neither the

parents not the physician were aware of any possible danger to the child and TTX was only discovered after an internet search by a neighbour (King, Hamilton & Kassutto, 2000). This indicates the lack of awareness not only by the general public but also by medical professionals, most likely due to the rare albeit deadly nature of this toxin.

4.6 Further Research Opportunities

This study leaves various opportunities for further research. The sample size from this study was small, with only three adult newts from Britain being tested for TTX using the LC-MS method. A larger sample size may find that some newts do in fact possess low levels of TTX. On the other hand, a large sample size indicating no TTX would add to the probability of TTX being absent from British newts. It would be interesting to conduct further testing of TTX on British newts, as well as those from France, as these have so far not been proven to contain TTX, despite the species found in Britain being shown to possess TTX in other countries (Yotsu-Yamashita et al., 2007). Levels of TTX can vary greatly within the same population (Lorentz et al., 2016) and so individual testing would be needed to determine levels in each egg. The methods for detecting and quantifying TTX should continue to be adapted and improved as new analytical equipment is developed, facilitating cost effective, yet reliable and sensitive methods.

Another interesting research aspect regarding TTX in newts, is the origins of TTX in terrestrial species. Whilst marine species are known to uptake TTX via their diet, origins of TTX in terrestrial species have been under discussion. The recent evidence of a bacterial origin of TTX in *T. granulosa* (Vaelli et al., 2020) generates further research opportunities. As many of the bacteria that produce TTX are known, this could provide a strong indicator as

to the probability of TTX in *L. vulgaris* and *L. helveticus* in Britain and France, if these bacteria are found in these locations. Further research could be conducted to ascertain the presence or absence of these bacteria. This could also provide the option of an alternative method to sampling the newt or eggs, as water could be analysed for their presence.

In addition, as puffer fish containing TTX are still eaten in Japan, and accidental consummation occurs worldwide, further research into a cure for TTX poisoning would be beneficial. More insight into how resistant animals such as the garter snake can withstand TTX is needed. The research into the use of TTX as a painkiller (L'Abbee, 2003) also has potential to be continued, with perhaps other medical uses being discovered.

Finally, further research could also be conducted into how TTX and the species that possess it are recognised within the fishing and food industry. This research would highlight areas that could be improved upon in the education of the public, potentially changing regulations and thus saving lives in the future.

4.7 Conclusion

This study aimed to detect levels of TTX in eggs and adult newt, using HPLC/UV/Vis/FLD and LC-MS methods. The initial methods that were trialled provided ambiguous results, potentially due to errors with the sample preparations or the set-up of the equipment. Contamination within the negative controlled cast further doubt on the reliability of the experiments. Initial sample preparation methods were also unsuccessful, however, following protocol stated in literature, a successful sample preparation method was then established. Further research included LC-MS which was more reliably and accurately

conducted. These results accurately predicted no TTX in all three samples that were tested. Genetic analysis determined that two of these individuals were *L. helveticus*. The third individual was not able to be identified and may have been either *L. helveticus* or *L. vulgaris*. The method of LC-MS used to detect TTX has been used previously with success, proving reliable, accurate and precise, as well as more ethical than the traditional mouse bioassay method. Therefore, these methods could be adopted for future research. More research is needed into the extent of species that contain TTX, as well as their range, in order to ensure the safety of humans from the potentially fatal TTX poisonings that occur.

5. References

Actor, J. K. (2012). Elsevier's Integrated Review Immunology and Microbiology (Second Edition). doi: 10.1016/B978-0-323-07447-6.00009-0

Amphibian and Reptile Conservation, Holmes, F. (2014). Amphibian Identification, Retrieved from https://freshwaterhabitats.org.uk/wp-content/uploads/2017/02/Amphibian_ID_Guide_-_ARG_UK-ARC_17_Mar_2014.pdf

Amphibiaweb. (2021). Amphibia Web, Retrieved from www.amphibiaweb.org

Arbuckle, K. (2015). Evolutionary Context of Venom in Animals. In P. Gopalakrishnakone & A. Malhotra (Eds.), *Evolution of Venomous Animals and Their Toxins* (pp. 1-23). doi: 10.1007/978-94-007-6727-0_16-1

Asakawa, M., Ito, K. & Kajihara, H. (2013). Highly toxic ribbon worm *Cephalothrix simula* containing tetrodotoxin in Hiroshima Bay, Hiroshima Prefecture, Japan. *Toxins, 5*, 376-395. doi: 10.3390/toxins5020376

Babik, W., Pabijan, M., Arntzen, J. W., Cogalniceanu, D., Durka, W. & Radwan, J. (2009). Long-term survival of a urodele amphibian despite depleted major histocompatibility complex variation, *Molecular Ecology, 18*(5), 769-781. doi: 10.1111/j.1365-294X.2008.04057.x

Babik,W., Branicki,W., Crnobrnja-Isailovic,J., Cogalniceanu,D., Sas,I., Olgun,K., Poyarkov,N.A., Garcia-Paris, M. & Arntzen, J.W. (2005). Phylogeography of two European newt species - discordance between mtDNA and morphology. *Molecular Ecology*, 14(8), 2475-2491

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A. & Furey, A. (2014). Tetrodotoxin: Chemistry, toxicity, source, distribution and detection. *Toxins, 6*(2), 993-755. doi: 10.3390/toxins6020693

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z. S., Litmanovich, M., … Eisenman, A. (2008). Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. *Toxicon, 52*(8), 964-968. doi: 10.1016/j.toxicon.2008.10.001

Bodero, M., Gerssen, A., Portier, L., Klijnstra, M. D., Hoogenboom, R. L. A. P., Guzman, L., … Bovee, T. F. H. (2018). A strategy to replace the mouse bioassay for detecting and identifying lipophilic marine biotoxins by combining the neuro-2a bioassay and LC-MS/MS analysis. *Marine Drugs, 16*(12), 501. doi: 10.3390/md16120501

Brady, L. D. & Griffiths, R. (1995). Effects of pH and aluminium on the growth and feeding behaviour of smooth and palmate larvae, *Ecotoxicology, 4*, 299-306.

Brodie Jr, E.D. (1968). Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. *Copeia, 1968*(2), 307–313

Brodie Jr, E.D., Hensel, J.L. & Johnson, J.A. (1974). Toxicity of the Urodele amphibians *Taricha*, *Notophthalmus*, *Cynops*, and *Paramesotriton (Salamandridae*). *Copeia, 1974*(2), 506–511

Brown, M. S. & Mosher, H. S. (1963). Tarichatoxin: Isolation and purification. *Science*, *140*(3564), 295-296. doi: 10.1126/science.140.3564.295

Bucciarelli, G. M., Li, A., Zimmer, R. K., Kats, L. B. & Green, D. B. (2014). Quantifying tetrodotoxin levels in the California newt using a non-destructive sampling method. *Toxicon, 80,* 87-93. doi: 10.1016/j.toxicon.2014.01.009

Bucciarelli, G. M., Green, D. B., Shaffer, H. B. & Kats, L. B. (2016). Individual fluctuations in toxin levels affect breeding site fidelity in a chemically defended amphibian. *Proceedings of the Royal Society B: Biological Sciences, 283*(1831), 20160468. doi: 10.1098/rspb.2016.0468

Buchwald, H. D., Durham, L., Fischer, H. G., Harada, R. & Furhman, F. A. (1964). Identity of tarichatoxin and tetrodotoxin. *Science, 143*(3605), 474-475

Campbell, K., Vilariño, N., Botana, L. M. & Elliott, C. T. (2011). A European perspective on progress in moving away from the mouse bioassay for marine-toxin analysis. *TrAC Trends in Analytical Chemistry, 30*(2), 239-253. doi: 10.1016/j.trac.2010.10.010

Cardall, B.L., Brodie Jr, E.D., Brodie III, E.D. & Hanifin, C.T. (2004). Secretion and regeneration of tetrodotoxin in the rough-skin newt (*Taricha granulosa*). *Toxicon, 44*(8), 933–938

Chen, X. W., Liu, H. X., Jin, Y. B., Li, S. F., Bi, X., Chung, S., … Jiang, Y. Y. (2011). Separation, identification and quantification of tetrodotoxin and its analogs by LC–MS without calibration of individual analogs. *Toxicon, 57*(6), 938-943. doi: 10.1016/j.toxicon.2011.03.011

Chulanetra, M., Sookrung, N., Srimanote, P., Indrawattana, N., Thanongsaksrikul, J., Sakolvaree, Y., … Chaicumpa, W. (2011). Toxic marine puffer fish in Thailand seas and tetrodotoxin they contained. *Toxins, 3*(10), 1249-1262. doi: 10.3390/toxins3101249

Chung, M. Y., Vu, S. H., López-Pujol, J., Herrando-Moraira, S., Son, S., Suh, G. U., … Chung, M. G. (2018). Comparison of genetic variation between northern and southern populations of *Lilium cernuum* (Liliaceae): Implications for Pleistocene refugia, *PLoS ONE, 13*(1), e0190520. doi: 10.1371/journal.pone.0190520

Cohen, N. J., Deeds, J. R., Wong, E. S., Hanner, R. H., Yancy, H. F., … Gerber, S. I. (2009). Public health response to puffer fish (Tetrodotoxin) poisoning from mislabeled product. *Journal of Food Protection, 72*(4), 810-817. doi: 10.4315/0362-028x-72.4.810

Covaciu-Marcov, S. D., Cicort-Lucaciu, A., Mitrea, I., Sas, I., Căuş, A. V. & Cupşa, D. (2010). Feeding of three syntopic newt species (*Triturus cristatus, Mesotriton alpestris* and *Lissotriton vulgaris*) from Western Romania. *North-Western Journal of Zoology, 6*(1), 95-108

Cuthill, I. C., Sanghera, N. S., Penacchio, O., Lovell, P.G. Ruxton, G. D. & Harris, J. M. (2016). Optimizing countershading camouflage. *Proceedings of the National Academy of Science of the United States of America, 113*(46), 13093–13097. doi: 10.1073/pnas.1611589113

Daly, J. W., Gusovsky, F., Myers, C. W., Yotsu-Yamashita, M. & Yasumoto, T. (1994). First occurrence of tetrodotoxin in a dendrobatid frog (*Colostethus inguinalis*), with further

reports for the bufonid genus Atelopus. *Toxicon, 3*(32), 279-285. doi: 10.1016/0041-0101(94)90081-7

Daly, J. W., Padgett, W. L., Saunders, R. L. & Cover Jr, J.F. (1997). Absence of tetrodotoxins in a captive raised riparian frog, *Atelopus varius*. *Toxicon, 35*(5), 705–709

Dao, H. V., Takata, Y., Sato, S., Fukuyo, Y. & Kodama, M. (2009). Frequent occurrence of the tetrodotoxin-bearing horseshoe crab *Carcinoscorpius rotundicauda* in Vietnam. *Fisheries Science, 75*(2), 435-438. doi: 10.1007/s12562-008-0041-5

Du Toit, M. H., Eggen, P. O., Kvittingen, L., Partalli, V. & Schmid, R. (2012). Normal- and reverse-phase paper chromatography of leaf extracts of dandelions. *Journal of Chemical Education, 89*(10), 1295-1296. doi: 10.1021/ed200851w

EFSA Panel on Contaminants in the Food Chain (CONTAM), Knutsen, H. K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., … Peterson, A. (2017). Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. *EFSA Journal, 15*(4), e04752. doi: 10.2903/j.efsa.2017.4752

Endler, J. A. (1991). Interactions between predators and prey. In J. R. Krebs & M. B. Davies (Eds.), *Behavioural Ecology An Evolutionary Approach* (pp 169-196). Oxford: Blackwell Scientific Publications.

Froglife. (2020). Frog Life, Retrieved from www.froglife.org

Fry, B. (2015). *Venomous Reptiles and Their Toxins: Evolution, Pathophysiology and Biodiscovery*. New York: Oxford University Press.

Gall, B. G., Brodie III, E. D. & Brodie Jr, E. D. (2011). Survival and growth of the caddisfly *Limnephilus flavastellus* after predation on toxic eggs of the Rough-skinned Newt (*Taricha granulosa*). *Canadian Journal of Zoology, 89*(6), 483. doi: 10.1139/Z11-015

Gall, B. G., Farr, A. A., Engel, S. G. A. & Brodie Jr, E. D. (2011). toxic prey and predator avoidane: Responses of toxic newts to chemical stimuli from a predator and injured conspecifics. *Northwestern Naturalist, 92,* 1-6. doi: 10.1898/10-22.1

Gall, B. G., Stokes, A. N., French, S. S., Brodie III, E. D. & Brodie Jr, E. D. (2012). Female newts (*Taricha granulosa*) produce tetrodotoxin laden eggs after long term captivity. *Toxicon, 60*(6), 1057-1062. doi: 10.1016/j.toxicon.2012.07.017

Gall, B. G., Stokes, A. N., French, S. S., Schlepphorst, E. A., Brodie III, E. D. & Brodie Jr, E. D. (2011). Tetrodotoxin levels in larval and metamorphosed newts (*Taricha granulosa*) and palatability to predatory dragonflies. *Toxicon, 57*(7-8), 978-983. doi: 10.1016/j.toxicon.2011.03.020

Gallacher, S. & Birkbeck, T. H. (1992). A tissue culture assay for direct detection of sodium channel blocking toxins in bacterial culture supernates. *FEMS Microbiology Letters, 92*(1), 101-107. doi: 10.1016/0378-1097(92)90549-4

Geffeney, S. L., Fujimoto, E., Brodie Jr, E. D., Brodie III, E. D. & Ruben, P. C. (2005). Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. *Nature, 434*(7034), 759-63. doi: 10.1038/nature03444

Griffiths, R. (1993). The effect of pH on feeding behaviour in newt larvae (Triturus : Amphibia), *Journal of Zoology, 231*, 285-290. doi: 10.1111/j.1469-7998.1993.tb01918.x

Griffiths, R. (1996). *Newts and Salamanders of Europe*, London: Academic Press

Griffiths, R. & de Wijer, P. (1994). Differential effects of pH and temperature on embryonic development in the British newts (Triturus), *Journal of Zoology, 234*, 613-622

Gross, J. H. (2004). Mass Spectrometry. doi: 10.1007/978-3-540-36756-7

Hamasaki, K., Kogure, K. & Ohwada, K. (1996). A biological method for the quantitative measurement of tetrodotoxin (TTX): Tissue culture bioassay in combination with a water-soluble tetrazolium salt. *Toxicon, 34*(4), 490-495. doi: 10.1016/0041-0101(95)00151-4

Hanifin, C. T. (2010). The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. *Marine Drugs, 8*, 577-593. doi: 10.3390/md8030577

Hanifin, C.T., Brodie III, E.D. & Brodie Jr, E.D. (2002). Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. *Toxicon, 40*(8), 1149–1153

Hanifin, C.T., Brodie III, E.D. & Brodie Jr, E.D. (2003). Tetrodotoxin levels in eggs of the rough-skin newt, *Taricha granulosa*, are correlated with female toxicity. *Journal of Chemical Ecology, 29*(8), 1729–1739

Hanifin, C.T., Brodie III, E.D., Brodie Jr, E.D. (2004). A predictive model to estimate total skin tetrodotoxin in the newt *Taricha granulosa*. *Toxicon*, *43*(3), 243–249

Hanifin, C.T., Brodie Jr, E.D. & Brodie III, E.D. (2008). Phenotypic mismatches reveal escape from arms-race coevolution. *PLoS Biol*ogy, *6*(3), e60

Hanifin, C.T., Yotsu-Yamashita, M., Yasumoto, T., Brodie III, E.D. & Brodie Jr, E.D. (1999). Toxicity of dangerous prey: variation of tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. *Journal of Chemical Ecology, 25*(9), 2161–2175

Harris, R. J. & Arbuckle, K. (2016). Tempo and mode of the evolution of venom and poison in tetrapods. *Toxins, 8*(7), 193. doi: 10.3390/toxins8070193

How, M. J. & Zanker, J. M. (2014). Motion camouflage induced by zebra stripes. *Zoology, 117*(3), 163-170. doi: 10.1016/j.zool.2013.10.004

Indumathi, S. M. & Khora, S. S. (2017). Toxicity assessment and screening of tetrodotoxin in the oblong blowfish (*Takifugu oblongus*) from the Tamil Nadu Coast of Bay of Bengal, India. *Asian Pacific Journal of Tropical Medicine, 10*(3), 278-284. doi: 10.1016/j.apjtm.2017.03.007

Isbister, G. K. & Kiernan, M. C. (2005). Neurotoxic marine poisoning. *The Lancet Neurology, 4*(4), 219-228. doi: 10.1016/S1474-4422(05)70041-7

Jehle, R. & Arntzen, J. W. (2000). Post-breeding migrations of newts (*Triturus cristatus* and *T. marmoratus*) with contrasting ecological requirements. *Journal of Zoology, 251*, 297-306. doi: 10.1111/j.1469-7998.2000.tb01080.x

Jehle, R., Bouma, P., Sztatecsny, M. & Arntzen, J. W. (2000). High aquatic niche overlap in the newts *Triturus cristatus* and *T. marmoratus* (Amphibia, Urodela). *Hydrobiologia, 437*, 149-155. doi: 10.1023/A:1026554907400

Jehle, R., Theismeier, B. & Foster, J. (2011). *The crested newt: a dwindling pond dweller,* Germany: Laurenti Verlag and British Herpetological Society

Jehle, R., Wilson, G.A., Arntzen, J.W. & Burke, T. (2005). Contemporary gene flow and the spatio-temporal genetic structure of subdivided newt populations (*Triturus cristatus, T. marmoratus*). *Journal of Evolutionary Biology*, 18, 619-628.Jellet, J. F., Marks, L. J., Stewart, J. E., Dorey, M. L., Watson-Wright, W. & Lawence, J. F. (1992). Paralytic Shellfish poison (saxitoxin family) bioassays: Automated endpoint determination and standardization of the in vitro tissue culture bioassay, and comparison with the standard mouse bioassay. *Toxicon, 30*(10), 1143-1156

Jesus, S. S. d., Ferreira, G. F., Maciel, M. R. W. & Filho, R. M. (2019). Biodiesel purification by column chromatography and liquid-liquid extraction using green solvents. *Fuel, 235*, 1123-1130. doi: 10.1016/j.fuel.2018.08.107

Ji, Y., Liu, Y., Gong, Q. L., Zhou, L. & Wang, Z. P. (2011). Toxicity of cultured puffer fish and seasonal variations in China. *Aquaculture Research, 42*(8), 1186-1195. doi: 10.1111/j.1365-2109.2010.02707.x

Johnson, P. T. J., Calhoun, D. M., Stokes, A. N., Susbilla, C. B., McDevitt-Gallas, T., Briggs, C. J., … de Roode, J. C. (2018). Of poisons and parasites—the defensive role of tetrodotoxin against infections in newts. *Journal of Animal Ecology, 87*(4), 1192-1204. doi: 10.1111/1365-2656.12816

Katikou, P. & Vlamis, A. (2017). Tetrodotoxins: recent advances in analysis methods and prevalence in European waters. *Current Opinion in Food Science, 18*, 1-6. doi: 10.1016/j.cofs.2017.09.005

Kazemi, B., Gamberale-Stille, G., Wåtz, T., Wiklund, C. & Leimar, O. (2018). Learning of salient prey traits explains Batesian mimicry evolution. *Evolution, 72*(3), 531-539. doi: 10.1111/evo.13418

Kim, Y.H., Brown, G.B., Mosher, H.S. & Fuhrman, F.A. (1975). Tetrodotoxin: Occurrence in atelopid frogs of Costa Rica. *Science*, *189*, 151–152

King, B. R., Hamilton, R. J. & Kassutto, Z. (2000). “Tail of Newt”: an unusual ingestion. *Paediatric Emergency Care, 16*(4), 268-269.

Kogure, K., Tamplin, M. L., Simidu, U. & Colwell, R. R. (1988). A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. *Toxicon, 26*(2), 191-197. doi: 10.1016/0041-0101(88)90171-7

Kotaki, Y. & Shimizu, Y. (1993). 1-Hydroxy-5,11-dideoxytetrodotoxin, the first N-hydroxy and ringdeoxy derivative of tetrodotoxin found in the newt *Taricha granulosa*. *Journal of the American Chemical Society, 115*(3), 827–830

Kudo, Y., Chiba, C., Konoki, K., Cho, Y. & Yotsu-Yomashita, M (2015). Confirmation of the absence of tetrodotoxin and its analogues in the juveniles of the Japanese fire-bellied newt, Cynops pyrrhogaster, captive-reared from eggs in the laboratory using HILIC-LC-MS. *Toxicon*, *101*, 101-105. doi: 10.1016/j.toxicon.2015.05.008

Kupfer, A. & Teunis, S. F. M. (2001). Field observations of anti-predator behaviour in three species of newt (Genus: *Triturus*). *Herpetological Journal, 11*(3), 117-119.

L'Abbee, M. (2003). Painkiller purified from venom: move over, morphine - here comes the puffer fish. *The Report, 30*(7), 42.

Lago, J., Rodríguez, L. P., Blanco, L., Vieites, J. M. & Cabado, A.G. (2015). Tetrodotoxin, an extremely potent marine neurotoxin: Distribution, toxicity, origin and therapeutical uses. *Marine Drugs, 13*(10), 6384-6406. doi: 10.3390/md13106384

Laschi, C. (2017). Helping robots blend into the background. *Science, 358*(6360), 169. doi: 10.1126/science.aao5345

Lehmen, E. M., Brodie Jr, E. D. & Brodie III, E. D (2004). No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*. *Toxicon, 44*(3), 243-249. doi: 10.1016/j.toxicon.2004.05.019

Leung, K. S. Y., Fong, B. M. W. & Tsoi, Y. K. (2011). Analytical challenges: Determination of tetrodotoxin in human urine and plasma by LC-MS/MS. *Marine Drugs, 9*(11), 2291-2303. doi: 10.3390/md9112291

Levenson, C.H. & Woodhull, A.M. (1979). The occurrence of a tetrodotoxin-like substance in the red spotted newt, *Notophthalmus viridescens. Toxicon*, *17*, 184–187

Ligabue-Braun, R., Verli, H. & Carlini, C. R. (2012). Venomous mammals: A review. *Toxicon, 59*(7-8), 680-695. doi: 10.1016/j.toxicon.2012.02.012

Lin, C. L., Wu, Y. J., Lin, W. F., Hsieh, C. H., Hsieh, Y. L. & Hwang, D. F. (2014). Seasonal characteristics and tetrodotoxin-producing ability of bacteria in gastropod *Oliva hirasei*. *Hydrobiologia, 741*(1), 23-32. doi: 10.1007/s10750-013-1769-1

Ling, S., Chen, Q. A., Zhang, Y., Wang, R., Jin, N., Pang, J. & Wang, S. (2015). Development of ELISA and colloidal gold immunoassay for tetrodotoxin detection based on monoclonal antibody. *Biosensors and Bioelectronics, 71*, 256-260. doi: 10.1016/j.bios.2015.04.049

Lorentz, M. N., Stokes, A. N., Rößler. D. C. & Lötters, S. (2016). Tetrodotoxin. *Current Biology, 26*(19), 870-872. doi: 10.1016/j.cub.2016.05.067

Man, C. N., Noor, N. M., Harn, G. L., Lajis, R. & Mohamad, S. (2010). Screening of tetrodotoxin in puffers using gas chromatography–mass spectrometry. *Journal of Chromatography A, 1217*(47), 7455-7459. doi: 10.1016/j.chroma.2010.09.064

Manger, R. L., Leja, L. S., Lee, S. Y., Hungerford, J. M. & Wekell, M. M. (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. *Analytical Biochemistry, 214*(1), 190-194. doi: 10.1006/abio.1993.1476

Mebs, D. & Schmidt, K. (1989). Occurrence of tetrodotoxin in the frog *Atelopus oxyrhynchus*. *Toxicon, 27*(7), 819–822

Mebs, D., Yotsu-Yamashita, M., Yasumoto, T., Lotters, S. & Schluter, A. (1995). Further report of the occurrence of tetrodotoxin in *Atelopus* species (family: *Bufonidae*). *Toxicon, 33*(2), 246–249

Miaud, C. (1993). Predation on newt eggs (*Triturus alpestris* and *T. helveticus*): identification of predators and protective role of oviposition behaviour. *Journal of Zoology, 231*(4). doi: 10.1111/j.1469-7998.1993.tb01939.x

Miró, A., O'Brien, D., Hall, J. & Jehle, R. (2017). Habitat requirements and conservation needs of peripheral populations: the case of the great crested newt (*Triturus cristatus*) in the Scottish Highlands. *Hydrobiologia*, 792 (1), 169-181

Mosher, H. S., Fuhrman, F. A., Buchwald, H. D. & Fischer, H. G. (1964). Tarichatoxin-tetrodotoxin: A potent neurotoxin. *Science, 144*(3622), 1100-1110.

Müller, F. (1878). Über die Vortheile der Mimicry bei Schmetterlingen, *Zoologischer Anzeiger, 1*, 54-5.

Myers, G.S. (1942). Notes on Pacific coast *Triturus*. *Copeia, 1942*(2), 77–82

Natural Resources Wales. (2020). Morfa Dyffryn National Nature Reserve, near Barmouth, Retrieved from https://naturalresources.wales/days-out/places-to-visit/north-west-wales/morfa-dyffryn-national-nature-reserve/?lang=en

Nelson, D. R., Nisani, Z., Cooper, A. M., Fox, G. A., Gren, E. C., Corbit, A. G. & Hayes, W. K. (2014). Poisons, toxungens, and venoms: redefining and classifying toxic biological secretions and the organisms that employ them. *Biological Reviews of the Cambridge Philosophical Society, 89*(2), 450-465. doi: 10.1111/brv.12062

New Scientist. (2020). We were warned – so why couldn't we prevent the coronavirus outbreak?, Retrieved from https://www.newscientist.com/article/mg24532724-700-we-were-warned-so-why-couldnt-we-prevent-the-coronavirus-outbreak/

Orizaola, G. & Florentino, B. (2003). Oviposition behaviour and vulnerability of eggs to predation in four newt species (Genus *Triturus*). *Herpetelogical Journal, 13*(3), 121-124

Pavelka, L. A., Kim, Y. H. & Mosher, H. S. (1977). Tetrodotoxin and tetrodotoxin-like compounds from the eggs of the Costa Rican frog. *Atelopus chiriquiensis*, *Toxicon, 15*, 135–139

Philips, B. L. & Shine, R. (2006). An invasive species induces rapid adaptive change in a native predator: cane toads and black snakes in Australia. *Proceedings of the Royal Society B: Biological Sciences, 273*(1593), 1545-1550. doi: 10.1098/rspb.2006.3479

Pires Jr, O.R., Sebben, A., Schwartz, E.F., Largura, S.W., Bloch Jr, C., Morales, R.A. & Schwartz, C.A. (2002). Occurrence of tetrodotoxin and its analogues in the Brazilian frog *Brachycephalus ephippium* (Anura: Brachycephalidae). *Toxicon, 40*(6), 761–766

Pires, O.R., Sebben, A., Schwartz, E.F., Bloch, C., Morales, R.A. & Schwartz, C.A. (2003). The occurrence of 11-oxotetrodotoxin, a rare tetrodotoxin analogue, in the Brachycephalidae frog *Brachycephalus ephippium*. *Toxicon, 42*(5), 563–566

Pires Jr., O. R., Sebben, A., Schwartz, E. F., Morales, R. A. V., Bloch Jr, C. & Schwartz, C. A. (2005). Further report of the occurrence of tetrodotoxin and new analogues in the Anuran family Brachycephalidae. *Toxicon, 45*(1), 73-79. doi: 10.1016/j.toxicon.2004.09.016

Puech, B., Batsalle, B., Roget, P., Turquet, J., Quod, J. P., Allyn, J., … Gaüzère, B. A. (2014). Family tetrodotoxin poisoning in Reunion Island (Southwest Indian Ocean) following the consumption of *Lagocephalus sceleratus* (Pufferfish). *Bulletin de la Société de Pathologie Exotique, 107*(2), 79-84. doi: 10.1007/s13149-014-0340-2.

Pyron, R. A. & Wiens, J. J. (2011). A large-scale phylogeny of amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution, 61*(2), 543-583. doi: 10.1016/j.ympev.2011.06.012

Rae, M., Miró, A., Hall, J., O'Brien, K. & O'Brien, D. (2019). Evaluating the validity of a simple citizen science index for assessing the ecological status of urban drainage ponds. *Ecological Indicators*, 98, 1-8.

Rannap, R., Lõhmus, A. & Linnamägi, M. (2012). Geographic variation in habitat requirements of two coexisting newt species in Europe. Acta zoologica Academiae Scientiarum Hungaricae, 58(1), 69-86

Reverté, S., Soliño, L., Carnicer, O., Diogène, J. & Campàs, M. (2014). Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays. *Marine Drugs, 12*(12), 5719-5763. doi: 10.3390/md12125719

Ritson-Williams, R., Yotsu-Yamashita, M. & Paul, V. J. (2006). Ecological functions of tetrodotoxin in a deadly polyclad flatworm. *Proceedings of the National Academy of Sciences of the United States of America, 103*(9), 3176-3179. doi: 10.1073/pnas.0506093103

Rocha, L. F. O. (2016). Analysis of molecular structures and mechanisms for toxins derived from venomous animals. *Computational Biology and Chemistry, 61,* 8-14. doi: 10.1016/j.compbiolchem.2015.11.004

Roşca, I., Gherghel, I., Strugariu, A. & Zamfirescu, Ş. R. (2013). Feeding ecology of two newt species (*Triturus cristatus* and *Lissotriton vulgaris*) during the reproduction season. *Knowledge and Management of Aquatic Ecosystems, 408*, 5. doi: 10.1051/kmae/2013040Published online15 April 2013

Saito, T., Maruyama, J., Kano, H., Zenigeshi, H., Noguchi, T., Harada, T., … Hashimoto, S. (1984). Toxicity and tetrodotoxin resistance of cultured tiger puffer. *Journal of Japan Fisheries Society, 50*(9), 1573-1575. doi: 10.2331/suisan.50.1573

Saito, T., Miura, N., Namera, A., Ota, S., Miyzaki, S. & Inokuchi, S. (2014). A rapid sample preparation procedure using MonoSpin CBA and amide columns for tetrodotoxin detection in serum and urine using LC–MS/MS Analysis. *Chromatographia, 77*(9-10), 687-693. doi: 10.1007/s10337-014-2663-3

Saito, T., Noguchi, T., Shida, Y., Abe, T. & Hashimoto, K. (1991). Screening of tetrodotoxin and its derivatives in puffer-related species. *J-STAGE, 57*(8), 1573-1577. doi: 10.2331/suisan.57.1573

Santiago, M. & Strobel, S. (2013). Chapter Twenty-Four - Thin Layer Chromatography. *Methods in Enzymology, 533*, 303-324. doi: 10.1016/B978-0-12-420067-8.00024-6

Schwenk, K. (1994). Why snakes have forked tongues. *Science, 263*(5153), 1573.

Sebben, A., Schwartz, C.A., Valente, D. & Mendes, E.G. (1986). A tetrodotoxin-like substance found in the Brazilian frog *Brachycephalus ephippium*. *Toxicon, 24*(6), 799–806

Servedio, M. R. (2007). The effects of predator learning, forgetting, and recognition errors on the evolution of warning coloration. *Evolution, 54*(3), 751-763. doi: 10.1111/j.0014-3820.2000.tb00077.x

Shalabai, V. V., Taranchenko, V. F., Rybal'chenko, I. V., Aksenov, A. V., Antokhin, A. M., Semchenko, F. M. & Krymchak, M. S. (2016). Use of high-performance liquid chromatography coupled with high-resolution mass spectrometry for the identification and quantitative determination of tetrodotoxin in pharmaceuticals. *Journal of Analytical Chemistry, 72*(6), 632-638. doi: 10.1134/S1061934817060144

Sheumack, D. D., Howden, M. E., Spence, I. & Quinn, R. J. (1978). Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. *Science, 199*(4325), 188-189. doi: 10.1126/science.619451

Shimizu, Y. & Kobayashi, M. (1983). Apparent lack of tetrodotoxin biosynthesis in captured *Taricha torosa* and *Taricha granulosa*. *Chemical and Pharmaceutical Bulletin (Tokyo), 31*, 3625–3631

Simidu, U., Noguchi, T., Hwang, D. F., Shida, Y. & Hashimoto, K. (1987). Marine bacteria which produce tetrodotoxin. *Applied and Environmental Microbiology, 53*(7), 1714-1715.

Skelhorn, J. & Rowe, C. (2006). Taste-rejection by predators and the evolution of unpalatability in prey. *Behavioral Ecology and Sociobiology 60*(4), 550-555. doi: 10.1007/s00265-006-0199-8

Sousounis, K., Qi, F., Yadav, M. C., Millán, J. L., Toyama, F., Chiba, C., … Tsonis, P. A. (2015). A robust transcriptional program in newts undergoing multiple events of lens regeneration throughout their lifespan. eLife, 4, 10.7554/eLife.09594

Speybroeck, J., Beukema, W., Dufresnes, C., Fritz, U., Jablonski, D., Lymberakis, P., Martinez-Solano, I., Razetti, E., Vamberger, M., Vences, M., Vörös, J., & Crochet, P.A. (2020). Species list of the European herpetofauna – 2020 update by the Taxonomic Committee of the Societas Europaea Herpetologica. Amphibia-Reptilia 41: 139-189

Stevens, M. & Merilaita, S. (2011). *Animal Camouflage: Mechanisms and Function*. Retrieved from https://ebookcentral-proquest-com.salford.idm.oclc.org/lib/salford/detail.action?docID=775017#

Stokes, A. N., Ducey, P. K., Neuman-Lee, L., Hanifin, C. T., French, S. S., Pfrender, M. E., Brodie III, E. D. & Brodie Jr, E. D. (2014). Confirmation and distribution of tetrodotoxin for the first time in terrestrial invertebrates: two terrestrial flatworm species (*Bipalium adventitium* and *Bipalium kewense*). *PLoS ONE, 9*(6), 100718. doi: 10.1371/journal.pone.0100718

Stokes, A. N., Williams, B. L. & French, S. S. (2012). An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. *Biological Procedures Online, 14*(1), 3. doi: 10.1186/1480-9222-14-3

Stommel, E. W. & Watters, M. R. (2004). Marine neurotoxins: Ingestible toxins. *Current Treatment Options in Neurology, 6*(2), 105-114.

Suenaga, K. & Kotoku, S. (1980). Detection of tetrodotoxin in autopsy material by gas chromatography. *Archives of Toxicology, 44*(4), 291-297. doi: 10.1007/BF00278036

Suleiman, M., Mohammad, J., Jelip, J., William, T. & Chua, T. H. (2017). An outbreak of tetrodotoxin poisoning from consuming horseshoe crabs in Sabah. *The Southeast Asian Journal of Tropical Medicine and Public Health, 48*(1), 197-203.

Suzuki, H. (2016). Differences in susceptibility of mouse strains to tetrodotoxin. *Toxicon, 119*, 168-170. doi: 10.1016/j.toxicon.2016.06.004

Tamele, I. J., Silva, M. & Vasconcelos, V. (2019). The incidence of tetrodotoxin and its analogs in the Indian Ocean and the Red Sea. *Marine Drugs, 17*(1), 28. doi: 10.3390/md17010028

Tanu, M. B., Mahmud, Y., Tsuruda, K., Arakawa, O. & Noguchi, T. (2001). Occurrence of tetrodotoxin in the skin of a rhacophoridid frog *Polypedates* sp from Bangladesh. *Toxicon, 39*(7), 937–941

Teramoto, N. & Yotsu-Yamashita, M. (2015). Selective blocking effects of 4,9-anhydrotetrodotoxin, purified from a crude mixture of tetrodotoxin analogues, on NaV1.6 channels and its chemical aspects. *Marine Drugs, 13*(2), 984-985. doi: 10.3390/md13020984

Tsuruda, K., Arakawa, O., Kawatsu, K., Hamano, Y., Takatani, T. & Noguchi, T. (2002). Secretory glands of tetrodotoxin in the skin of the Japanese newt *Cynops pyrrhogaster*. *Toxicon*, *40*(2), 131–136

Tsuruda, K., Arakawa, O., Noguchi, T. (2001). Toxicity and toxin profiles of the newt, *Cynops pyrrhogaster* from western Japan. *Journal of Natural Toxins, 10*(2), 79–89

Vaelli, P. M., Theis, K. R., Williams, J. E., O'Connell, L. A., Foster, J. A. & Eisthen, H. L. (2020). The skin microbiome facilitates adaptive tetrodotoxin production in poisonous newts. *ELife, 9*, e53898. doi: 10.7554/eLife.53898

Verrell, P. A. & Francillon, H. (1986). Body size, age and reproduction in the smooth newt, *Triturus vulgaris*. *Journal of Zoology, 210*(1), 89-100. doi: 10.1111/j.1469-7998.1986.tb03622.x

Vogt, W. (1970). What is a toxin? *Toxicon, 8*(3), 251. doi: 10.1016/0041-0101(70)90211-4

Wakely, J.F., Fuhrman, G.J., Fuhrman, F.A., Fischer, H.G. & Mosher, H.S. (1966). The occurrence of tetrodotoxin (tarichatoxin) in amphibia and the distribution of the toxin in the organs of newts (*Taricha*). *Toxicon, 3*(3), 195–203

Williams, B. L. (2010). Behavioral and chemical ecology of marine organisms with respect to tetrodotoxin. *Marine Drugs, 8*, 381-398. doi: 10.3390/md8030381

Williams, B. L., Hanifin, C. T., Brodie Jr, E. D. & Brodie II, E. D. (2010). Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*). *Chemoecology, 20*(4), 285-290. doi: 10.1007/s00049-010-0057-z

Williams, B. L., Hanifin, C. T., Brodie Jr, E. D. & Brodie II, E. D. (2012). Predators usurp prey defenses? Toxicokinetics of tetrodotoxin in common garter snakes after consumption of rough-skinned newts. *Chemoecology, 22*(3), 179-185. doi: 10.1007/s00049-011-0093-3

Winandy, L., Legrand, P. & Denoël, M. (2017). Habitat selection and reproduction of newts in networks of fish and fishless aquatic patches. *Animal Behaviour, 123*, 107-115. doi: 10.1016/j.anbehav.2016.10.027

Wixom, R. L., Gehrke, C. W., Berezkin, V. G. & Janak, J. (2010). Chromatography – A new discipline of science. In R. L. Wixom & C. W. Gehrke (Eds.), *Chromatography – A science of discovery* (pp. 1-13). New Jersey: John Wiley & Sons, Incorporated.

Worsfold, P. J. (2019). Spectrophotometry Overview, in Worsfold, P., Poole, C., Townshend, A. & Miró, M. (Eds.) *Encyclopedia of Analytical Science (Third Edition)* (pp.244-248). Retrieved from 10.1016/B978-0-12-409547-2.14265-9

Yan, Q., Yu, P. H. F. & Li, H. Z. (2005). Detection of tetrodotoxin and bacterial production by *Serratia marcescens, World Journal of Microbiology and Biotechnology, 21*(6), 1255-1258. doi: 10.1007/s11274-005-1926-4

Yasumoto, T., Yotsu, M. & Murata, M. (1988). New tetrodotoxin analogues from the newt *Cynops ensicauda*. *Journal of the American Chemical Society, 110*, 2344

Yong-Yi, S., Lu, L., Gui-Sheng, L., Robert, W. M. & Ya-Ping, Z. (2012). Parallel evolution of auditory genes for echolocation in bats and toothed whales. *PLoS Genetics, 8*(6). doi: 10.1371/journal.pgen.1002788

Yotsu, M., Iorizzi, M., Yasumoto, T. (1990). Distribution of tetrodotoxin, 6-epitetrodotoxin, and 11-deoxytetrodotoxin in newts. *Toxicon, 28*(2), 238–241

Yotsu-Yamashita, M. & Mebs, D. (2001). The levels of tetrodotoxin and its analogue 6-epitetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*. *Toxicon, 39*(8), 1261–1263

Yotsu-Yamashita, M. & Mebs, D. (2003). Occurrence of 11-oxotetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*, and further studies on the levels of tetrodotoxin and its analogues in the newt's efts. *Toxicon, 41*(7), 893–897

Yotsu-Yamashita, M., Mebs, D. & Flachsenberger, W. (2007). Distribution of tetrodotoxin in the body of the blue-ringed octopus (*Hapalochlaena maculosa*). *Toxicon, 49*(3), 410-412. doi: 10.1016/j.toxicon.2006.10.008

Yotsu-Yamashita, M., Mebs, D., Kwet, A. & Schneider, M. (2007). Tetrodotoxin and its analogue 6-epitetrodotoxin in newts (*Triturus* spp.; Urodela, Salamandridae) from southern Germany. *Toxicon, 50*(2), 306–309

Yotsu-Yamashita, M., Mebs, D. & Yasumoto, T. (1992). Tetrodotoxin and its analogues in extracts from the toad *Atelopus oxyrhynchus* (family: Bufonidae). *Toxicon, 30*(11), 1489-1492. doi: 10.1016/0041-0101(92)90526-B

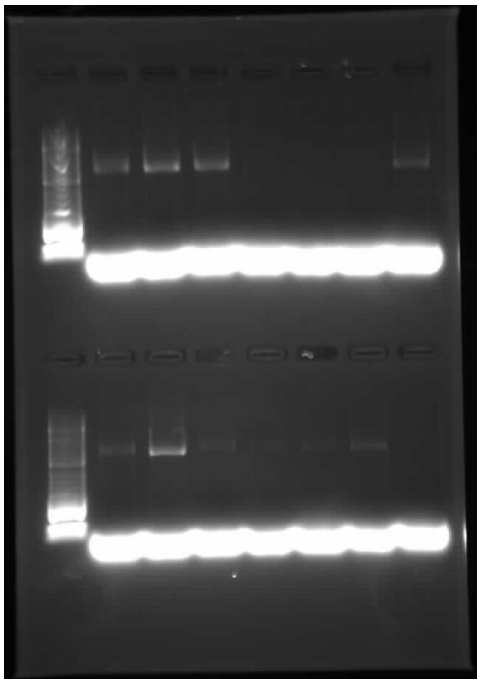
Yotsu-Yamashita, M. & Tateki, E. (2010). First report on toxins in the Panamanian toads *Atelopus limosus*, *A. glyphus* and *A. certus. Toxicon, 55*(1), 153–156

Yotsu-Yamashita, M., Toennes, S. W. & Mebs, D. (2017). Tetrodotoxin in Asian newts (*Salamandridae*). *Toxicon, 134*, 14-17. doi: 10.1016/j.toxicon.2017.05.014

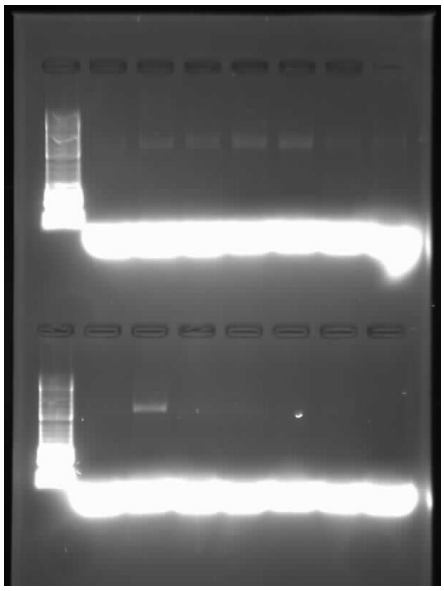
Zhou, Y., Li, Y., Lu, S., Ren, H., Li, Z., Zhang, Y., … Liu, Z. (2010). Gold nanoparticle probe-based immunoassay as a new tool for tetrodotoxin detection in puffer fish tissues. *Sensors and Actuators B: Chemical, 146*(1), 368-372. doi: 10.1016/j.snb.2010.02.049

6. Appendix

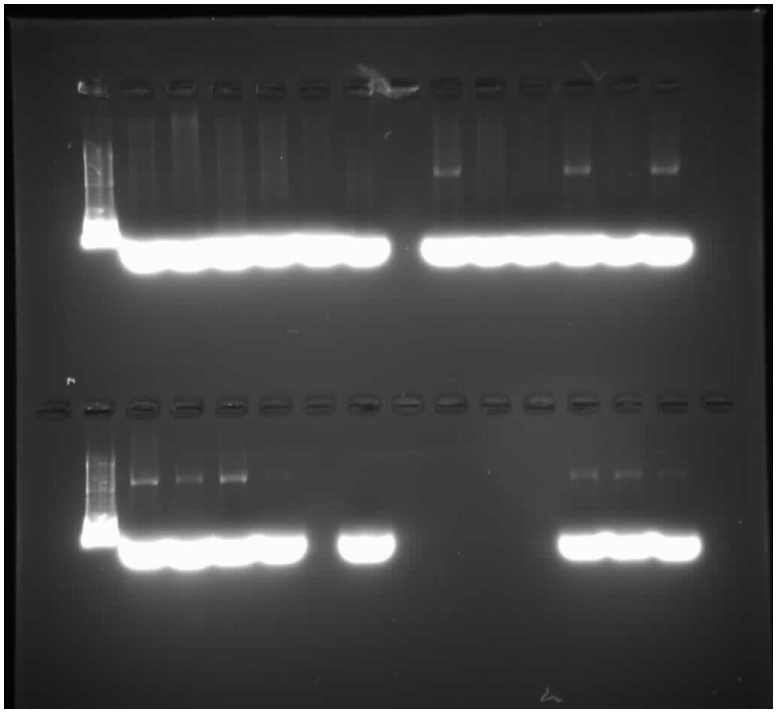
6.1 Gels



Appendix 1. PCR products from diluted DNA, samples 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14 & negative control



Appendix 2. PCR products from diluted DNA second run, 20 µl reaction volume, samples 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14 & negative control



Appendix 3. PCR products samples 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 4x, 5x, 7x & negative control followed by 4x, 5x, 7x from previous PCR

6.2 Sample Preparation Records

Appendix 4. Sample preparation methods using acetonitrile with varying tissue types, sonic temperatures and sonic run

Sample Number	Newt Number	Tissue Type	Weight (g)	Sonic Run Time (mins)	Sonic Temperature (˚C)
1	N2	Hand	0.0062	15	Room temp
2	N2	Tail tip	0.0137	15	Room temp
3	N2	Foot	0.0184	15	Room temp
4	N2	Dorsal skin	0.0198	15	Room temp
5	N2	Ventral skin	0.0885	15	Room temp
6	N1	Hand	0.004	15	Room temp
7	N1	Tail tip	0.0134	15	Room temp
8	N1	Foot	0.028	15	Room temp
9	N1	Dorsal skin	0.0116	15	Room temp
10	N1	Ventral skin	0.0112	15	Room temp
1-10 X	Negative control			15	Room temp
1-10 Y	Negative control			15	Room temp
11	N2	Hand	0.0062	15	50
12	N2	Tail tip	0.0137	15	50
13	N2	Foot	0.0184	15	50
14	N2	Dorsal skin	0.0198	15	50
15	N2	Ventral skin	0.0885	15	50
16	N1	Hand	0.004	15	50
17	N1	Tail tip	0.0134	15	50
18	N1	Foot	0.028	15	50
19	N1	Dorsal skin	0.0116	15	50
20	N1	Ventral skin	0.0112	15	50
11-20 X	Negative control			15	50
11-20 Y	Negative control			15	50
21	N3	Ventral + dorsal skin	0.0716	15	Room temp
22	N3	Front + back leg	0.0355	15	Room temp
23	N3	Tail	0.0574	15	Room temp
24	N4	Ventral + dorsal skin	0.0776	15	Room temp
25	N4	Front + back leg	0.0306	15	Room temp
26	N4	Tail	0.0913	15	Room temp
27	N5	Ventral + dorsal skin	0.0531	15	Room temp
28	N5	Front + back leg	0.029	15	Room temp
29	N5	Tail	0.0625	15	Room temp

21-29 X	Negative control			15	Room temp
21-29 Y	Negative control			15	Room temp
30	N3	Ventral + dorsal skin	0.0716	15	50
31	N3	Front + back leg	0.0355	15	50
32	N3	Tail	0.0574	15	50
33	N4	Ventral + dorsal skin	0.0776	15	50
34	N4	Front + back leg	0.0306	15	50
35	N4	Tail	0.0913	15	50
36	N5	Ventral + dorsal skin	0.0531	15	50
37	N5	Front + back leg	0.029	15	50
38	N5	Tail	0.0625	15	50
30-38 X	Negative control			15	50
30-38 Y	Negative control			15	50
39	N6	Tail tip	0.0129	30	Room temp
40	N6	Dorsal skin	0.0222	30	Room temp
39-40	Negative control			30	Room temp
41	N6	Tail tip	0.0129	30	50
42	N6	Dorsal skin	0.0222	30	50
41-42	Negative control			30	50

Appendix 5. Sample preparation of newts using acetic acid and varying tissue types

Sample Number	Newt Number	Tissue Type	Weight (g)	Centrifuge Time	Centrifuge RPM
43	N7	Dorsal skin	0.0624	30	13,300
44	N7	Dorsal skin	0.1641	30	13,300
45	N7	Tail	0.2497	30	13,300
46	N7	Intestines	0.2851	30	13,300
47	N7	Whole newt		30	13,300
48	N7	Whole newt		30	13,300
49	N7	Whole newt		30	13,300
50	N7	Whole newt		30	13,300
X 43-50	Negative control			30	13,300
Y 43-50	Negative control			30	13,300

6.3 HPLC/UV/Vis Results

Appendix 6. Results of newt samples prepared using acetonitrile run on the HPLC/UV/Vis spectrometer at 250nm

Sample Number	Peak Number	Time	Area	Height	Width	Area%	Symmetry
Blank	1	6.872	295.8	9.9	0.3909	23.93	2.447
	2	10.043	335.3	26.2	0.1673	27.128	3.711
	3	10.261	154.9	14	0.1733	12.533	0.482
	4	12.668	450.1	33.4	0.199	36.409	5.038
1	1	3.428	41.7	4.7	0.1437	3.452	1.042
	2	4.147	59.3	4.3	0.2176	4.901	0.74
	3	9.712	186.2	8.2	0.2794	15.396	9.958
	4	10.061	480.4	24.5	0.2528	39.729	1.011
	5	11.05	17.5	1.7	0.1413	1.448	2.518
	6	11.833	35.6	2.5	0.193	2.94	1.404
	7	12.206	17.6	1.8	0.1562	1.453	1.319
	8	13.741	42.7	2.3	0.2441	3.528	2.201
	9	14.78	15.8	1.3	0.171	1.306	2.627
	10	16.45	15.3	1.1	0.2324	1.265	0.923
	11	17.915	83.7	4.2	0.2645	6.918	3.161
	12	24.504	91.2	2.3	0.5145	7.543	2.602
	13	25.321	97.7	3.9	0.4036	8.079	0.837
	14	30.817	24.7	2.7	0.1373	2.04	1.368
2	1	2.26	10.7	2.3	0.0763	0.32	2.781
	2	2.358	9.9	2.1	0.0741	0.296	0.63
	3	2.646	550.9	32.5	0.234	16.471	0.407
	4	3.148	77.5	24.4	0.0511	2.318	0.974
	5	3.278	76.3	19.2	0.0623	2.28	0.776
	6	3.424	17.6	4.4	0.0646	0.527	0.647
	7	3.53	21.3	1.9	0.1384	0.636	0.0702
	8	9.865	175.4	7.2	0.299	5.243	6.81
	9	10.215	500.3	32	0.2164	14.959	1.625
	10	11.154	18.5	1.9	0.1436	0.552	1.888
	11	11.879	318	20	0.2156	9.509	2.366
	12	12.331	13.3	1.3	0.1535	0.396	1.033
	13	13.138	9.9	1.1	0.1428	0.297	1.692
	14	13.524	29.7	2.7	0.1665	0.889	1.112
	15	15.05	94.4	4.1	0.303	2.824	2.992
	16	16.243	79.5	4	0.274	2.378	2.741
	17	16.981	13.6	1.4	0.1386	0.405	3.713
	18	17.57	1060.3	65.7	0.2222	31.703	1.917
	19	19.385	16.5	1.3	0.1919	0.492	2.461
	20	19.644	18.5	1.9	0.1549	0.555	0.642
	21	21.773	36.6	2	0.272	1.095	0.828
	22	24.029	76.3	3.3	0.3514	2.283	1.179

	23	29.237	119.5	3.7	0.4619	3.572	1.176
3	1	2.592	43.5	6.6	0.111	1.113	0.764
	2	3.204	10.9	3	0.0569	0.278	1.756
	3	3.687	9.2	1.2	0.1211	0.235	0.405
	4	9.86	170.2	7.4	0.2814	4.354	6.012
	5	10.21	457.9	29.3	0.2166	11.714	1.633
	6	11.156	19.6	2.1	0.1372	0.502	1.86
	7	11.883	468.7	28.4	0.2245	11.99	2.486
	8	12.336	15.1	1.6	0.149	0.387	0.96
	9	13.141	18.7	1.9	0.1467	0.479	1.477
	10	13.529	39.3	3.7	0.159	1.005	1.081
	11	15.062	193.9	8.2	0.3127	4.959	2.831
	12	16.152	30.1	1.3	0.3037	0.77	2.464
	13	16.988	28.2	2.8	0.1428	0.72	3.696
	14	17.583	2269.6	141.1	0.2216	58.059	1.883
	15	19.401	26.3	2.2	0.1768	0.673	2.225
	16	19.663	34.9	3.6	0.1553	0.892	0.666
	17	21.356	73.1	2.2	0.4311	1.87	3.604
4	1	2.613	87.2	13.8	0.1017	2.257	0.905
	2	2.837	34.2	3.9	0.1175	0.885	0.296
	3	3.212	25.5	9.1	0.0467	0.66	1.291
	4	3.301	53.2	7.9	0.0906	1.378	0.234
	5	9.859	169.5	7.2	0.2883	4.386	6.161
	6	10.209	448.7	29.6	0.2107	11.614	1.761
	7	11.15	32.2	3.2	0.1462	0.834	1.764
	8	11.875	495.5	31.4	0.2164	12.825	2.372
	9	12.33	22	2.3	0.1499	0.57	1.059
	10	13.144	15	1.6	0.1422	0.388	1.529
	11	13.522	49.6	4.6	0.1626	1.283	1.084
	12	15.049	199.5	8.9	0.2961	5.164	2.676
	13	16.145	34.3	1.5	0.3086	0.887	2.243
	14	16.974	64	4.7	0.188	1.657	4.032
	15	17.57	2048	130.6	0.2189	53.005	1.832
	16	19.385	27.7	2.5	0.1687	0.716	2.123
	17	19.647	45.5	4.5	0.1604	1.178	0.665
	18	21.331	12.1	1.2	0.1582	0.312	0.524
5	1	2.613	87.2	13.8	0.1017	2.257	0.905
	2	2.837	34.2	3.9	0.1175	0.885	0.296
	3	3.212	25.5	9.1	0.0467	0.66	1.291
	4	3.301	53.2	7.9	0.0906	1.378	0.234
	5	9.859	169.5	7.2	0.2883	4.386	6.161
	6	10.209	448.7	29.6	0.2107	11.614	1.761
	7	11.15	32.2	3.2	0.1462	0.834	1.764
	8	11.875	495.5	31.4	0.2164	12.825	2.372
	9	12.33	22	2.3	0.1499	0.57	1.059

	10	13.144	15	1.6	0.1422	0.388	1.529
	11	13.522	49.6	4.6	0.1626	1.283	1.084
	12	15.049	199.5	8.9	0.2961	5.164	2.676
	13	16.145	34.3	1.5	0.3086	0.887	2.243
	14	16.974	64	4.7	0.188	1.657	4.032
	15	17.57	2048	130.6	0.2189	53.005	1.832
	16	19.385	27.7	2.5	0.1687	0.716	2.123
	17	19.647	45.5	4.5	0.1604	1.178	0.665
	18	21.331	12.1	1.2	0.1582	0.312	0.524
6	1	2.413	27.1	4.8	0.0817	0.604	1.261
	2	2.627	7.6	2	0.0582	0.169	2.159
	3	3.053	723.2	27.6	0.3473	16.133	1.249
	4	3.661	10.8	1.5	0.1067	0.24	0.451
	5	9.859	145.1	6.5	0.274	3.238	6.265
	6	10.204	441.6	29.8	0.207	9.85	1.734
	7	11.155	18.5	1.9	0.1397	0.413	2.564
	8	11.876	584.4	32.2	0.2421	13.036	2.713
	9	13.148	14.8	1.6	0.1375	0.329	1.932
	10	13.512	27.2	2.9	0.1448	0.607	0.683
	11	15.048	247.1	9.3	0.347	5.512	2.502
	12	16.129	93.6	3.1	0.4221	2.089	1.7
	13	16.979	45.5	1.8	0.3223	1.015	13.766
	14	17.57	1613.7	98.7	0.2268	35.998	2.31
	15	17.908	74.1	6.8	0.1729	1.652	0.369
	16	19.382	26.6	2	0.1931	0.594	2.676
	17	19.643	36.5	3.5	0.1651	0.815	0.617
	18	20.776	29.6	2	0.2273	0.66	1.755
	19	22.803	74.5	2.1	0.4513	1.661	0.677
	20	25.328	71.9	3.1	0.3768	1.604	1.068
	21	28.992	169.4	5	0.4938	3.78	0.865
7	1	2.585	36.7	5.8	0.1002	1.044	0.67
	2	3.241	21.6	4.6	0.0691	0.613	1.432
	3	3.37	21.5	2.3	0.1467	0.612	0.236
	4	3.671	11	1.4	0.1125	0.312	1.016
	5	9.864	163.4	7.2	0.2823	4.647	6.059
	6	10.213	410.6	27.7	0.2048	11.676	1.838
	7	11.156	26.2	2.7	0.1438	0.746	1.711
	8	11.878	404.7	25.6	0.2141	11.508	2.361
	9	12.332	16.1	1.7	0.1442	0.458	0.911
	10	13.144	16.2	1.8	0.1443	0.462	1.485
	11	13.524	40.4	3.8	0.1597	1.149	1.052
	12	15.057	171.2	7.5	0.3043	4.869	2.725
	13	16.989	33.6	3.3	0.145	0.955	3.194
	14	17.584	2043.1	130.7	0.2184	58.094	1.805
	15	19.401	24	2.1	0.1715	0.684	2.034
	16	19.663	33.7	3.5	0.1551	0.959	0.666

	17	21.348	42.7	1.8	0.3176	1.213	2.541
8	1	2.553	10.4	2.5	0.0669	0.288	1.402
	2	2.938	6.4	1.2	0.0791	0.178	2.313
	3	3.116	32.8	5.6	0.0909	0.906	1.245
	4	3.306	32.8	2.8	0.1684	0.905	0.374
	5	3.66	13.6	1.9	0.1066	0.377	0.37
	6	9.864	176.9	7.5	0.2908	4.89	6.176
	7	10.216	434.8	28.3	0.2133	12.016	1.784
	8	11.154	18	1.7	0.1514	0.498	2.482
	9	11.874	488.8	26.7	0.2437	13.508	2.65
	10	13.136	14.2	1.4	0.1546	0.393	1.625
	11	13.519	29.8	2.6	0.1702	0.824	1.094
	12	15.055	170.6	6.2	0.3575	4.713	3.29
	13	17.585	2140.9	97.6	0.2918	59.159	2.498
	14	19.399	27.3	1.9	0.2104	0.753	2.615
	15	19.661	21.4	2.2	0.1596	0.592	0.571
9	1	2.432	18.3	3.1	0.0851	0.28	1.154
	2	2.689	12.3	3.4	0.0583	0.189	1.262
	3	2.831	23.7	5.8	0.0657	0.363	2.495
	4	2.999	559.3	37.9	0.2001	8.553	0.339
	5	3.507	54.9	6.1	0.1195	0.839	0.544
	6	3.776	10.4	1.5	0.0944	0.158	0.339
	7	9.878	169.8	7.6	0.2786	2.597	7.982
	8	10.204	481.4	32.1	0.2087	7.363	1.709
	9	11.146	11.6	1.2	0.1593	0.178	3.599
	10	11.535	41.1	4.9	0.1223	0.628	2.539
	11	11.87	311.6	27	0.1647	4.766	1.583
	12	13.112	93.3	5.3	0.2409	1.427	2.935
	13	13.514	38.4	3.5	0.1641	0.587	1.118
	14	15.048	419.4	18.1	0.3087	6.414	2.61
	15	16.13	80.3	3	0.3496	1.228	2.535
	16	16.979	192.9	12.5	0.2107	2.95	2.266
	17	17.575	3619.8	248.9	0.2056	55.362	1.499
	18	19.653	294.6	10.8	0.3628	4.506	2.585
	19	21.336	84.8	3.5	0.3246	1.297	2.201
	20	23.104	20.5	1.4	0.2258	0.314	0.78
10	1	2.387	12.9	1.6	0.1078	0.355	5.202
	2	2.588	48.5	11.6	0.0668	1.34	1.092
	3	2.874	296.1	18.7	0.2046	8.174	0.512
	4	3.255	23	2.7	0.1128	0.635	0.172
	5	9.87	197.3	8.4	0.2989	5.445	6.388
	6	10.212	449.8	29.2	0.2138	12.416	1.748
	7	11.151	14.1	1.5	0.1337	0.39	2.275
	8	11.872	449.3	24.1	0.2474	12.402	2.798
	9	13.127	26.6	1.5	0.2401	0.735	3.26

	10	13.517	26.1	2.4	0.1621	0.72	1.131
	11	15.049	133.4	4.8	0.3591	3.682	3.562
	12	17.577	1906.8	86.7	0.2927	52.635	2.485
	13	19.387	22.9	1.5	0.2199	0.632	2.81
	14	19.648	15.9	1.6	0.1559	0.439	0.57
1-10 Negative Control X	1	2.99	762	28.9	0.344	17.291	0.821
	2	3.67	11.3	1.4	0.1179	0.256	0.55
	3	9.866	200.1	8.2	0.2994	4.54	6.913
	4	10.207	444	28.9	0.2134	10.075	1.687
	5	11.153	21.5	2.4	0.1324	0.487	1.907
	6	11.881	544.7	32.9	0.2234	12.361	2.592
	7	12.333	16.3	1.7	0.1467	0.37	0.918
	8	13.135	26.4	2	0.1851	0.6	2.228
	9	13.522	39.5	3.7	0.1596	0.897	1.063
	10	15.057	184.6	7.6	0.3209	4.189	3.037
	11	16.991	30.1	2.3	0.1759	0.683	4.648
	12	17.585	2041.1	126.4	0.2224	46.318	1.911
	13	19.398	24.3	2	0.1866	0.551	2.208
	14	19.66	25.8	2.7	0.1542	0.585	0.642
	15	21.347	35.1	1.5	0.3103	0.797	2.485
1-10 Negative Control Y	1	3.265	51.3	5.5	0.1223	1.448	4.46
	2	3.376	18.6	2.8	0.1111	0.525	0.299
	3	3.665	22.9	1.9	0.1634	0.646	2.63
	4	9.863	180.9	7.6	0.2906	5.11	6.474
	5	10.209	405.6	27	0.2094	11.456	1.761
	6	11.15	22.1	2.4	0.1352	0.625	1.726
	7	11.875	413.2	26.2	0.2139	11.672	2.388
	8	12.328	17.2	1.8	0.1513	0.486	0.899
	9	13.137	17.6	1.9	0.1458	0.498	1.39
	10	13.519	37.8	3.5	0.1602	1.067	1.048
	11	15.054	196.2	8.4	0.3094	5.542	2.827
	12	16.144	31.5	1.5	0.2721	0.89	2.568
	13	16.984	63.5	5.6	0.16	1.794	2.946
	14	17.579	1960.8	126.6	0.2166	55.382	1.841
	15	19.395	35	2.9	0.1822	0.988	2.185
	16	19.658	41.7	4.3	0.1544	1.178	0.66
	17	21.112	14	1	0.201	0.396	2.509
	18	21.346	10.5	1.1	0.1558	0.298	0.459
11	1	3.236	224.4	10.1	0.3123	7.048	1.127
	2	9.863	206.9	8.6	0.2961	6.497	7.544
	3	10.207	431.3	27.6	0.2161	13.541	1.681
	4	11.15	23.7	2.4	0.1419	0.745	1.709
	5	11.877	376	23.7	0.2169	11.806	2.362
	6	12.331	15.8	1.7	0.148	0.495	0.892
	7	13.14	14.2	1.5	0.1493	0.447	1.515

	8	13.523	36.1	3.4	0.1603	1.132	1.066
	9	15.058	136	6	0.2998	4.269	2.749
	10	16.986	35.7	3	0.1631	1.12	3.491
	11	17.584	1596.6	103.3	0.2144	50.132	1.804
	12	19.401	22.2	1.9	0.1745	0.696	2.073
	13	19.664	29.2	3	0.1547	0.917	0.667
	14	21.355	36.8	1.6	0.31	1.156	2.442
12	1	2.517	8.2	1.1	0.1035	0.483	0.713
	2	3.062	47.5	3.6	0.1777	2.814	0.288
	3	3.602	21	2.5	0.1405	1.243	0.69
	4	3.866	30.5	5	0.0937	1.81	1.143
	5	3.979	5.7	2	0.0477	0.336	0.333
	6	9.883	271.4	11	0.307	16.078	8.837
	7	10.217	438.9	27.9	0.2172	26.002	1.971
	8	11.149	8.9	1.1	0.1197	0.528	1.11
	9	11.535	11.4	1.4	0.1278	0.676	1.072
	10	11.88	117.9	11.9	0.1491	6.983	0.783
	11	13.127	13	1.3	0.1576	0.772	1.147
	12	13.528	15.8	1.8	0.1372	0.938	0.972
	13	15.063	37.2	3.5	0.1585	2.204	0.717
	14	16.985	24.9	2.9	0.1277	1.475	1.535
	15	17.59	610.9	65.2	0.1413	36.196	0.74
	16	19.678	24.7	2.4	0.1613	1.462	0.719
13	1	3.989	10.5	3.9	0.0436	1.511	0.809
	2	4.423	437.7	10	0.6178	62.945	0.852
	3	10.098	53	6.7	0.1244	7.621	4.185
	4	10.321	52.3	4.3	0.1643	7.521	2.789
	5	11.88	11	1	0.1612	1.579	0.607
	6	17.59	75.8	7.5	0.1497	10.907	0.64
	7	19.396	55	4.2	0.1893	7.916	0.582
14	1	2.779	6.5	1.7	0.0606	0.22	1.235
	2	3.331	161.7	11.5	0.1783	5.477	0.889
	3	9.864	170.5	7.4	0.2817	5.776	7.033
	4	10.209	416.4	27.6	0.2099	14.106	1.654
	5	11.153	17.4	1.9	0.1338	0.588	1.719
	6	11.877	327.1	20.7	0.2146	11.083	2.411
	7	12.329	12.8	1.4	0.1467	0.435	0.852
	8	13.143	14.4	1.5	0.1485	0.489	1.634
	9	13.524	38.6	3.6	0.161	1.308	1.091
	10	15.055	134.4	5.9	0.304	4.553	2.77
	11	16.149	33.3	1.6	0.2815	1.126	1.963
	12	16.983	54.7	4.7	0.1638	1.853	2.827
	13	17.578	1504.9	99.9	0.2097	50.983	1.777
	14	19.389	25.1	2.2	0.1715	0.85	2.007
	15	19.652	34	3.5	0.1555	1.153	0.669

15	1	2.632	5.4	1.4	0.063	0.209	0.866
	2	2.985	398	13.3	0.3678	15.333	0.33
	3	9.865	186.2	8.1	0.2843	7.173	7.074
	4	10.21	425.2	27.5	0.2142	16.382	1.652
	5	11.155	11.4	1.2	0.1352	0.439	1.839
	6	11.877	230.2	14.2	0.2193	8.869	2.386
	7	13.52	82.4	3.7	0.2974	3.173	3.184
	8	15.054	78.1	3.1	0.3256	3.008	2.972
	9	16.306	34	1.1	0.4056	1.31	5.997
	10	17.582	1115	64.8	0.2368	42.956	1.565
	11	19.394	14.4	1.1	0.1961	0.553	2.507
	12	19.659	15.4	1.6	0.156	0.594	0.655
16	1	2.448	25.6	8.5	0.049	0.688	1.914
	2	2.533	30.1	9.1	0.0544	0.81	0.823
	3	2.659	9.6	2.7	0.0598	0.258	0.82
	4	2.976	1317.7	62.3	0.2666	35.452	0.54
	5	9.863	171.6	7.3	0.2893	4.617	7.829
	6	10.21	439.7	28.8	0.2142	11.831	1.684
	7	11.154	12.2	1.4	0.1281	0.327	2.298
	8	11.876	479	27.6	0.2324	12.888	2.524
	9	13.505	16.3	1.8	0.1394	0.438	0.689
	10	15.047	130.2	5	0.3414	3.503	2.422
	11	17.575	941	56	0.2299	25.317	2.39
	12	17.911	32.1	3	0.1714	0.864	0.329
	13	19.38	16.8	1.1	0.2179	0.453	2.898
	14	19.647	14.4	1.5	0.1547	0.388	0.646
	15	29.025	80.5	2.4	0.4824	2.165	0.87
17	1	3.571	20.4	2.3	0.1267	1.588	0.746
	2	3.795	30.1	4.9	0.0932	2.346	0.668
	3	9.877	267.2	10.9	0.3039	20.83	8.316
	4	10.212	428	27.2	0.2134	33.37	2.021
	5	11.876	101.8	9.6	0.1577	7.935	0.917
	6	12.342	9.1	1	0.1439	0.712	0.892
	7	13.524	15.5	1.7	0.1463	1.206	1.064
	8	15.057	22.4	2	0.1683	1.744	0.824
	9	16.98	17.7	1.8	0.1468	1.377	1.84
	10	17.584	355.3	36.7	0.1471	27.701	0.755
	11	19.669	15.3	1.5	0.1598	1.19	0.729
18	1	3.191	207.1	8.8	0.2849	7.081	1.302
	2	9.87	219.2	9.1	0.3057	7.495	7.073
	3	10.213	443.8	28.2	0.2176	15.17	1.736
	4	11.874	232.7	13.2	0.2364	7.954	2.585
	5	13.144	11.2	1.1	0.1582	0.382	1.867
	6	13.52	29.1	2.6	0.1651	0.996	1.165

	7	15.05	94.3	3.5	0.3545	3.225	3.571
	8	17.579	1653.6	77.8	0.2818	56.529	2.569
	9	19.394	16.9	1.2	0.2014	0.576	2.723
	10	19.651	17.3	1.7	0.1579	0.591	0.609
19	1	2.929	27.3	3.2	0.1181	1.144	2.003
	2	3.193	30.1	1.1	0.3426	1.259	0.0268
	3	9.868	202.9	8.5	0.3041	8.5	6.867
	4	10.214	421.1	27.3	0.2116	17.636	1.785
	5	11.156	12.2	1.4	0.1312	0.511	1.992
	6	11.877	311.4	18	0.232	13.044	2.564
	7	13.516	24.8	2.2	0.1648	1.037	1.125
	8	15.051	86.4	3.3	0.3411	3.62	3.449
	9	17.576	1259.3	60	0.281	52.747	2.37
	10	19.645	12	1.2	0.1549	0.503	0.601
20	1	2.616	12.4	2.9	0.0672	0.442	1.097
	2	2.923	34.4	2.9	0.1669	1.226	2.108
	3	3.289	45.1	4.7	0.1237	1.608	0.608
	4	9.865	174.9	8	0.2712	6.242	6.687
	5	10.211	430.7	28	0.2134	15.369	1.701
	6	11.154	10.7	1.2	0.1294	0.383	1.824
	7	11.875	275.3	16.3	0.2294	9.824	2.474
	8	13.138	12.3	1.1	0.1679	0.439	1.834
	9	13.521	26.2	2.4	0.1637	0.935	1.111
	10	15.055	101.5	4	0.3307	3.622	3.224
	11	17.584	1646.1	83.7	0.2653	58.74	2.254
	12	19.397	15.7	1.2	0.1961	0.56	2.539
	13	19.657	17.1	1.7	0.1543	0.61	0.624
11-20 Negative Control X	1	3.266	38.7	3.6	0.1502	1.205	2.587
	2	3.389	13.5	2.7	0.0793	0.42	0.305
	3	9.865	181	7.8	0.2838	5.641	7.459
	4	10.211	412.9	27.2	0.2089	12.867	1.805
	5	11.152	16	1.8	0.1297	0.498	1.711
	6	11.876	371.2	23	0.2182	11.566	2.419
	7	13.133	18.9	1.9	0.1547	0.59	1.414
	8	13.521	31	3	0.1576	0.965	1.004
	9	15.057	159.6	6.7	0.3134	4.974	2.908
	10	16.99	24.4	2.5	0.1379	0.761	3.267
	11	17.583	1828.3	115.8	0.2201	56.971	1.847
	12	19.397	21.7	1.8	0.1802	0.677	2.222
	13	19.657	29.9	3.1	0.1531	0.931	0.669
	14	21.337	62.1	1.9	0.4259	1.934	3.735
11-20 Negative Control Y	1	3.279	131.4	8.9	0.1867	5.412	1.692
	2	9.864	182.1	8	0.2833	7.503	7.301
	3	10.212	404.5	26.7	0.209	16.665	1.801

	4	11.155	15.3	1.7	0.1329	0.632	1.748
	5	11.877	349.7	22	0.2172	14.408	2.33
	6	13.138	12.4	1.3	0.1495	0.511	1.551
	7	13.521	27.4	2.6	0.1608	1.128	1.037
	8	15.051	108.8	4.7	0.3108	4.483	2.844
	9	16.987	20.6	1.6	0.1754	0.848	4.446
	10	17.578	1140.1	71.8	0.2212	46.967	1.834
	11	19.391	16.1	1.3	0.1797	0.664	2.135
	12	19.654	18.9	1.9	0.1555	0.779	0.657
21	1	3.598	19.2	1.3	0.199	0.74	0.892
	2	4.243	34.2	3.9	0.1224	1.32	3.159
	3	15.11	736.7	26.1	0.3852	28.433	2.854
	4	16.022	32.7	1.5	0.3589	1.261	1.624
	5	18.311	84.3	4.5	0.296	3.253	1.275
	6	19.862	16	1.1	0.2237	0.618	1.578
	7	20.442	16.5	1.2	0.2124	0.637	1.978
	8	20.901	82.1	6	0.2222	3.169	1.094
	9	22.623	48.6	2	0.3375	1.875	2.209
	10	23.541	104.6	4.8	0.32	4.037	1.655
	11	24.472	1202.3	79	0.2436	46.407	1.028
	12	25.134	169.3	9.2	0.2888	6.536	0.804
	13	27.586	44.4	2.7	0.2642	1.714	0.815
22	1	4.276	550.6	8.7	0.7789	18.474	0.428
	2	15.083	576.6	13.4	0.5697	19.347	4.374
	3	18.275	90.1	3.3	0.3914	3.023	2.196
	4	20.886	123.2	4.6	0.3773	4.133	2.728
	5	23.525	87.9	3.8	0.3322	2.949	1.761
	6	24.448	1425.6	70	0.3036	47.834	1.438
	7	27.564	58.7	1.9	0.4255	1.971	2.924
	8	28.104	31.6	1.7	0.2922	1.06	0.846
	9	28.861	36	1.5	0.3409	1.209	0.991
23	1	4.254	592.8	10.7	0.702	22.707	0.402
	2	15.083	666.1	17.2	0.5167	25.514	3.909
	3	18.271	91.2	3.4	0.3886	3.495	2.176
	4	20.877	91.2	3.8	0.3391	3.492	2.254
	5	23.516	101.3	4	0.3564	3.882	1.858
	6	24.446	1009.3	50.1	0.3009	38.661	1.443
	7	27.55	58.7	2.2	0.3799	2.25	1.678
24	1	3.44	14.7	1.6	0.1239	0.467	1.58
	2	3.722	8.9	1.1	0.1108	0.284	3.267
	3	4.328	578.4	13.7	0.5349	18.419	0.554
	4	15.075	662.5	16.6	0.5326	21.095	3.938
	5	18.284	77.4	3.3	0.3539	2.463	1.77
	6	20.877	89.4	3.5	0.3606	2.848	2.659

	7	22.471	167.7	4.7	0.4727	5.34	1.284
	8	23.518	44.5	1.9	0.3213	1.416	2.961
	9	24.45	1158.4	53.2	0.3201	36.888	1.81
	10	25.117	268.7	13	0.3209	8.557	0.946
	11	27.591	44.2	1.8	0.3633	1.406	1.658
	12	29.622	25.6	1.2	0.2746	0.815	1.436
25	1	4.372	563.8	9.1	0.744	20.308	0.636
	2	15.071	527.3	11.7	0.5911	18.992	4.544
	3	18.264	76.1	2.9	0.3803	2.741	2.376
	4	20.875	82.9	3.3	0.3472	2.987	2.415
	5	23.524	66.2	2.8	0.3396	2.383	1.861
	6	24.444	1460.1	70.5	0.3077	52.589	1.474
26	1	4.299	564.9	11.3	0.649	20.238	0.432
	2	15.084	658.9	17.2	0.5038	23.607	3.976
	3	18.269	94.7	3.6	0.3867	3.393	2.114
	4	20.885	101.5	4.2	0.3375	3.635	2.314
	5	23.529	112.5	4.6	0.3483	4.032	1.888
	6	24.456	1180.3	58.7	0.3005	42.287	1.459
	7	27.563	78.3	2.6	0.4074	2.807	2.037
27	1	3.486	16.4	2.1	0.1235	0.594	2.584
	2	3.802	19.1	2.1	0.134	0.693	1.019
	3	4.339	380.2	11.6	0.4233	13.807	0.393
	4	15.07	638.8	15.7	0.5412	23.195	4.072
	5	18.256	90.9	3.3	0.3996	3.302	2.113
	6	20.856	98.2	3.5	0.3851	3.565	3.09
	7	22.337	18.8	1.5	0.2075	0.682	2.141
	8	23.471	78.4	3	0.3678	2.846	2.202
	9	24.424	1043.6	52.6	0.2995	37.895	1.533
	10	25.099	341.2	17	0.3098	12.39	1.121
	11	29.586	28.4	1.3	0.3336	1.032	1.599
28	1	3.486	18.3	1.2	0.2035	0.845	0.798
	2	4.356	579.7	9.1	0.7797	26.784	0.63
	3	15.068	541.1	12	0.5912	24.998	4.608
	4	18.255	69.8	2.7	0.3823	3.225	2.187
	5	20.872	57.5	2.4	0.3418	2.658	2.305
	6	23.509	57.3	2.2	0.364	2.646	1.88
	7	24.446	840.7	40.6	0.3076	38.842	1.452
29	1	4.36	598.5	9.9	0.7505	26.82	0.665
	2	15.073	572	13	0.5827	25.632	4.336
	3	18.261	71.4	2.7	0.3778	3.198	2.037
	4	20.871	69	2.8	0.354	3.091	2.424
	5	23.503	73.1	2.8	0.3567	3.277	1.657
	6	24.439	847.6	42.8	0.299	37.982	1.355

21-29 Negative Control X	1	4.426	7.1	1.1	0.1063	0.502	2.84
	2	4.667	140.3	3.8	0.5239	9.903	0.201
	3	15.098	390.7	7.6	0.652	27.577	5.753
	4	18.281	43.8	2	0.3181	3.093	1.589
	5	20.904	41.2	2.1	0.2856	2.907	1.979
	6	23.55	53.9	2.2	0.3423	3.804	1.195
	7	24.466	696.2	40.7	0.2697	49.146	1.115
	8	27.59	43.5	1.6	0.3845	3.067	1.854
21-29 Negative Control Y	1	4.339	25.6	1.2	0.3131	1.649	4.765
	2	15.087	435.3	8.5	0.6618	27.989	5.313
	3	18.279	59.2	2.2	0.388	3.806	2.194
	4	20.885	50.6	2.1	0.346	3.251	2.351
	5	23.535	48.2	2.1	0.3323	3.1	1.893
	6	24.453	936.4	46.1	0.3031	60.205	1.456
30	1	3.628	51.4	3.6	0.1896	1.799	1.265
	2	4.318	288.3	13.4	0.2808	10.091	4.481
	3	4.488	14	2.4	0.0929	0.49	0.23
	4	15.104	592.5	17.6	0.4465	20.737	3.54
	5	18.326	74.1	3.6	0.3274	2.592	1.462
	6	19.855	20.2	1.4	0.2251	0.706	1.489
	7	20.899	119.7	5.7	0.3031	4.19	2.181
	8	22.431	38	1.5	0.3425	1.332	0.556
	9	23.547	153.9	7	0.3128	5.388	1.386
	10	24.466	1284.3	78.7	0.2603	44.948	1.131
	11	25.125	124.5	6.6	0.2925	4.356	0.831
	12	27.58	96.4	3.8	0.3742	3.373	1.686
31	1	4.264	78.6	2.4	0.4234	2.659	3.5
	2	4.541	100.1	1.3	0.9475	3.385	0.0264
	3	15.075	618.4	14.8	0.556	20.922	4.013
	4	18.271	99.1	3.7	0.3865	3.354	2.071
	5	20.883	142.4	5.1	0.3874	4.816	2.611
	6	23.535	136.1	5.8	0.3375	4.604	1.655
	7	24.455	1634.7	81.9	0.2989	55.305	1.404
	8	27.57	146.4	4	0.4994	4.954	2.697
32	1	4.262	598.6	11.3	0.6845	25.196	0.432
	2	15.078	615.9	14.9	0.5502	25.926	4.039
	3	18.269	79.2	3	0.3755	3.336	2.082
	4	20.883	71.1	2.9	0.3415	2.994	2.265
	5	23.528	83.6	3.3	0.3537	3.518	1.816
	6	24.459	869.2	43.6	0.2988	36.587	1.416
	7	27.572	58	2.1	0.3988	2.443	1.799
33	1	3.425	12.5	2.3	0.0852	0.353	1.279

	2	4.327	840.4	19.5	0.5385	23.674	0.893
	3	15.072	648.8	15.8	0.5472	18.275	4.075
	4	18.297	76	3.2	0.3576	2.142	1.878
	5	20.877	120	4.6	0.3612	3.38	2.786
	6	22.408	108.8	2.5	0.5571	3.065	1.35
	7	23.525	87.9	4	0.3193	2.475	2.254
	8	24.45	1407.3	69.5	0.3021	39.644	1.543
	9	25.118	165.9	8.5	0.3016	4.673	0.94
	10	27.572	82.3	2.8	0.4073	2.319	2.056
34	1	4.429	540.5	9.4	0.6996	24.587	0.759
	2	15.072	492.6	10.3	0.624	22.409	4.835
	3	18.26	69.3	2.6	0.3768	3.152	2.2
	4	20.875	71	3	0.3348	3.231	2.359
	5	23.518	74.5	3	0.3496	3.39	1.769
	6	24.441	897.5	44.7	0.302	40.827	1.421
	7	27.552	52.9	2	0.3759	2.405	1.657
35	1	4.363	683.8	11.9	0.7036	21.766	0.811
	2	15.073	675.1	16.8	0.536	21.489	3.872
	3	18.266	106.5	4	0.3808	3.391	2.166
	4	20.874	146.3	5.5	0.3718	4.657	2.577
	5	23.524	111.1	4.5	0.3431	3.536	1.771
	6	24.451	1340.3	67.8	0.2966	42.664	1.406
	7	27.56	78.5	2.7	0.4038	2.498	1.95
36	1	3.586	53.4	4.1	0.1771	1.817	1.44
	2	4.327	565.2	12.8	0.5415	19.242	0.603
	3	15.084	669	17	0.524	22.777	3.881
	4	18.281	90	3.1	0.4075	3.064	2.418
	5	20.873	99.8	3.7	0.377	3.398	2.803
	6	22.361	80.8	2.1	0.5111	2.751	1.028
	7	23.509	99.2	3.9	0.3601	3.378	2.039
	8	24.439	1077.2	53.7	0.302	36.672	1.519
	9	25.109	135	6.9	0.3037	4.595	1.082
	10	27.541	67.7	2.4	0.4066	2.306	1.798
37	1	4.246	52.7	2.2	0.325	2.511	3.74
	2	4.49	120.9	2.3	0.6446	5.761	0.0569
	3	15.063	455.2	9	0.658	21.695	4.99
	4	18.256	67.9	2.7	0.3676	3.235	2.2
	5	20.853	71	2.9	0.3447	3.385	2.31
	6	23.496	61.7	2.7	0.334	2.939	1.773
	7	24.411	1213.9	59.8	0.3047	57.857	1.431
	8	27.527	54.9	2	0.3925	2.617	1.772
38	1	3.462	15.7	1.4	0.1577	0.693	0.484
	2	4.254	61.5	1.7	0.4626	2.717	8.126

	3	15.083	609.9	15.2	0.5324	26.96	4.005
	4	18.283	84.4	3.2	0.3806	3.729	2.222
	5	20.883	97.2	3.9	0.3487	4.295	2.456
	6	23.521	88.9	3.6	0.3485	3.931	1.822
	7	24.445	1230.5	61.2	0.3007	54.394	1.433
	8	27.556	74.2	2.6	0.4081	3.281	1.941
30-38 Negative Control X	1	4.519	491.9	8.6	0.6896	27.404	0.916
	2	15.081	454.8	9.3	0.6305	25.333	5.096
	3	18.274	58.9	2.2	0.3891	3.281	2.168
	4	20.884	46.7	1.9	0.3377	2.601	2.419
	5	23.52	43.6	1.7	0.3748	2.428	2.057
	6	24.451	699.3	34.1	0.3052	38.953	1.442
30-38 Negative Control Y	1	4.267	520.3	8.4	0.7845	25.098	0.348
	2	15.07	492.4	10.4	0.6125	23.749	4.82
	3	18.263	70.4	2.7	0.3763	3.396	2.012
	4	20.883	58.2	2.4	0.3471	2.807	2.36
	5	23.528	62.9	2.6	0.3434	3.036	1.801
	6	24.454	869	42.6	0.3062	41.914	1.438
39	1	4.338	86.4	2.7	0.4326	4.235	2.976
	2	15.071	617.3	14.8	0.5491	30.251	4.101
	3	18.269	76.3	2.9	0.375	3.738	2.279
	4	20.871	88.6	3.3	0.3696	4.34	2.628
	5	23.515	76.7	3.1	0.3503	3.757	1.828
	6	24.443	1095.4	53.1	0.3086	53.68	1.454
40	1	3.53	14.8	1.7	0.1306	0.483	1.073
	2	4.287	728.8	14.3	0.6276	23.82	0.668
	3	15.087	628.9	15	0.5539	20.557	4.05
	4	18.273	84.2	3.3	0.3717	2.753	1.83
	5	20.875	99.7	3.7	0.3697	3.26	2.926
	6	23.507	90.6	3.2	0.3901	2.961	1.937
	7	24.437	1264.7	65.5	0.2893	41.338	1.437
	8	25.1	83.3	4.2	0.3062	2.724	0.88
	9	27.534	64.4	2.4	0.3876	2.105	1.718
39-40 Negative Control	1	4.284	40.7	2.3	0.2565	2.747	2.294
	2	15.081	481.5	9.9	0.6373	32.532	5.008
	3	18.277	57.4	2.2	0.3799	3.882	2.17
	4	20.875	49.6	2.1	0.3432	3.353	2.366
	5	23.506	44.7	1.7	0.3632	3.017	1.862
	6	24.437	806.2	40.1	0.3005	54.47	1.447
41	1	4.654	410.4	9	0.5878	26.01	0.922
	2	15.095	423.8	8.7	0.6251	26.858	5.426
	3	18.274	46.3	2	0.3258	2.934	1.757

	4	20.888	40.7	2	0.2937	2.578	1.967
	5	23.532	53.6	2.2	0.3361	3.397	1.371
	6	24.449	603.1	34.6	0.2714	38.223	1.179
42	1	3.465	9.6	1.3	0.1163	0.329	1.687
	2	4.307	716.5	12.3	0.7331	24.69	0.74
	3	15.082	599.9	13.7	0.5792	20.673	4.285
	4	18.268	100.8	3.2	0.446	3.472	2.645
	5	20.876	89.7	3.9	0.3321	3.092	2.279
	6	23.518	113.8	4.5	0.3542	3.923	1.776
	7	24.442	1154.4	59.3	0.2951	39.782	1.416
	8	25.103	36.3	2	0.2752	1.252	0.858
	9	27.547	80.9	2.7	0.419	2.786	2.009
41-42 Negative Control	1	4.508	346.5	7.8	0.5497	24.851	0.255
	2	15.104	343.4	5.5	0.7784	24.625	7.076
	3	18.284	33.5	1.6	0.3106	2.4	1.626
	4	20.899	29.8	1.5	0.2879	2.14	2.107
	5	23.552	43	1.9	0.3167	3.086	1.255
	6	24.46	562.4	33.7	0.2625	40.338	1.123
	7	27.579	35.7	1.3	0.3727	2.561	1.71

Appendix 7. Results of newt samples prepared using acetic acid run on HPLC/UV/Vis spectrometer at 250nm

Sample Number	Peak Number	Time	Area	Height	Width	Area %	Symmetry
43	1	3.131	1923.8	91.1	0.3324	7.231	1.031
	2	4.414	12553.6	453.7	0.3528	47.182	1.262
	3	5.239	8513.3	431.4	0.2642	31.996	0.508
	4	5.773	41.5	11.2	0.0633	0.156	0.678
	5	6.065	1199.5	146	0.1419	4.508	0.83
	6	6.306	900.7	170.3	0.0857	3.385	0.971
	7	6.532	378.5	62.3	0.0969	1.423	0.684
	8	7.209	83.1	6.5	0.188	0.312	0.774
	9	8.591	57.2	4.2	0.2095	0.215	0.848
	10	12.56	31.4	1.9	0.2685	0.118	1.136
	11	13.055	27.4	1.4	0.3152	0.103	0.59
	12	16.462	719.6	22.4	0.499	2.705	1.05
	13	25.74	57.7	2.4	0.3489	0.217	0.988
	14	26.379	11.9	1.1	0.1705	0.045	0.629
	15	27.176	13.5	1.1	0.1977	0.051	0.932
	16	28.076	94.5	3.6	0.3745	0.355	1.182
44	1	3.126	1054.3	55.1	0.313	8.163	0.762
	2	4.286	261.5	15.1	0.2219	2.025	83.614

	3	4.458	3180.4	367.3	0.135	24.624	0.94
	4	4.806	5580.3	434.5	0.1938	43.205	0.676
	5	5.66	782.2	104.4	0.1294	6.056	1.228
	6	5.82	572.2	153	0.0576	4.43	0.774
	7	6.064	37.4	11.2	0.0549	0.29	1.365
	8	6.25	654.4	69.9	0.1533	5.066	0.842
	9	7.165	104.6	5.9	0.2437	0.81	0.605
	10	8.561	20	1.4	0.218	0.155	0.788
	11	12.54	16.4	1	0.2614	0.127	1.253
	12	13.105	43.2	2.1	0.3216	0.335	0.841
	13	16.432	473.3	14.2	0.5068	3.665	1.058
	14	28.093	135.6	5.3	0.3664	1.05	0.77
45	1	3.035	795.4	40.5	0.3072	1.758	0.982
	2	4.419	9430.1	629.4	0.1908	20.841	2.715
	3	4.668	3457.4	398.2	0.1233	7.641	0.405
	4	5.28	21316.2	945.8	0.2969	47.11	0.343
	5	5.787	69.4	25	0.0463	0.153	0.34
	6	6.053	6096.9	635.1	0.144	13.475	0.693
	7	6.314	2186.5	292.1	0.1113	4.832	0.393
	8	7.193	216	14.6	0.2108	0.477	0.675
	9	8.588	22.7	1.3	0.2393	0.05	0.975
	10	11.954	41.4	1.8	0.3367	0.092	1.348
	11	12.566	32.4	2.1	0.245	0.072	1.116
	12	13.163	178	8.5	0.3297	0.393	0.883
	13	16.475	948.5	29.7	0.4891	2.096	1.089
	14	27.188	35	1.7	0.3052	0.077	2.188
	15	28.026	421.5	14.7	0.4224	0.931	0.82
46	1	4.169	155.5	52.9	0.0463	1.696	3.228
	2	4.222	26.9	14.2	0.0321	0.293	1.001
	3	4.329	312.7	63.9	0.0831	3.409	0.927
	4	4.597	8032.8	552.3	0.2037	87.581	0.498
	5	5.893	479.6	22.4	0.2756	5.229	0.625
	6	7.112	22.7	1.6	0.2228	0.248	0.997
	7	16.377	141.6	4.3	0.4707	1.544	1.025
47	1	2.811	3837.2	244.3	0.2393	11.118	0.711
	2	3.309	19.1	2	0.1476	0.055	0.2
	3	4.378	667.8	80.6	0.1127	1.935	10.591
	4	4.446	919.5	176.4	0.0748	2.664	0.328
	5	4.678	869.8	188.2	0.0759	2.52	1.112
	6	4.887	4524.5	498.8	0.1358	13.109	0.677
	7	5.273	15336.9	985.6	0.2195	44.436	0.499
	8	5.683	1425.6	269.6	0.0857	4.13	0.75
	9	5.855	1963.7	466.1	0.0652	5.69	0.909

	10	5.962	167.1	48.1	0.0545	0.484	0.235
	11	6.329	2309.2	147.3	0.2109	6.691	0.255
	12	6.74	173.9	32	0.0833	0.504	0.963
	13	7.193	18.9	2.5	0.1172	0.055	1.545
	14	7.356	19.7	2.6	0.1204	0.057	0.335
	15	8.579	50	3.4	0.2255	0.145	0.791
	16	12.559	49.9	2.9	0.2775	0.145	0.999
	17	13.175	98.9	5.1	0.3071	0.286	0.867
	18	16.47	1165.3	35.2	0.5043	3.376	1.086
	19	23.282	180.7	2.9	0.8135	0.524	4.597
	20	25.764	131.1	4.7	0.4105	0.38	1.149
	21	27.183	37.6	2.2	0.2591	0.109	1.646
	22	28.086	61.2	3	0.2864	0.177	0.867
	23	30.457	58.5	3.3	0.2499	0.169	0.653
	24	33.198	26.7	1.6	0.2391	0.077	0.698
	25	33.917	98.2	5.8	0.2491	0.285	0.618
	26	34.87	303.2	16.9	0.2677	0.879	0.672
48	1	2.813	4046.4	239.3	0.2513	11.051	0.61
	2	4.382	611.6	59	0.153	1.67	21.88
	3	4.445	1231.2	236.7	0.0747	3.363	0.373
	4	4.665	903.5	200	0.0727	2.468	0.833
	5	4.886	5653.7	677.7	0.1273	15.441	0.701
	6	5.285	18222	1194.4	0.216	49.767	0.538
	7	5.665	794.1	136.3	0.096	2.169	0.409
	8	5.839	1777.2	426.8	0.0646	4.854	0.874
	9	5.956	233	72.2	0.0517	0.636	0.429
	10	6.329	511.2	75.5	0.115	1.396	0.959
	11	6.502	397.3	58.3	0.1035	1.085	0.415
	12	6.709	56.9	10.9	0.0808	0.155	0.196
	13	7.191	20.5	2.5	0.1266	0.056	1.831
	14	7.359	20.8	2.8	0.1182	0.057	0.396
	15	8.575	44.4	3.1	0.2169	0.121	0.743
	16	12.557	48.9	2.9	0.2823	0.134	1.007
	17	13.168	102.2	5.3	0.3061	0.279	0.84
	18	16.459	1035.8	31.5	0.4954	2.829	1.084
	19	23.28	181	2.8	0.8259	0.494	4.95
	20	25.77	128.6	4.5	0.4053	0.351	1.182
	21	27.184	22.1	1.8	0.204	0.06	1.024
	22	28.081	65.2	3.3	0.2843	0.178	0.802
	23	30.452	58.3	3.2	0.2505	0.159	0.643
	24	33.199	30.6	1.8	0.2493	0.084	0.652
	25	33.915	107.3	6	0.2581	0.293	0.571
	26	34.867	310.6	17.4	0.2661	0.848	0.667
49	1	2.816	3411.4	211.3	0.2404	9.49	0.595

	2	4.383	671.8	67.5	0.1481	1.869	18.244
	3	4.449	1235.9	245.1	0.0728	3.438	0.394
	4	4.669	984.8	213.7	0.0738	2.74	0.883
	5	4.888	5816.4	710.4	0.1255	16.18	0.724
	6	5.291	18546.4	1233.8	0.2134	51.592	0.566
	7	5.671	609.5	121.1	0.0847	1.696	0.577
	8	5.825	1220	308.4	0.0622	3.394	0.78
	9	5.952	263	80	0.0524	0.732	0.472
	10	6.329	804.8	109.5	0.1098	2.239	0.629
	11	6.529	147.6	20.8	0.1047	0.411	0.0969
	12	7.194	30.8	3.1	0.1461	0.086	2.255
	13	7.35	16.4	2.1	0.1229	0.046	0.259
	14	8.587	173.6	13.2	0.2029	0.483	0.84
	15	12.564	55	3	0.2899	0.153	0.906
	16	13.172	20.3	1.1	0.2819	0.056	0.692
	17	16.471	1225.6	38.5	0.4872	3.409	1.016
	18	19.27	97.7	2.5	0.489	0.272	1.011
	19	25.774	153.8	6.2	0.3759	0.428	1.06
	20	27.186	23.3	1.7	0.2135	0.065	0.923
	21	30.459	66	3.8	0.2442	0.184	0.628
	22	33.924	88.5	4.3	0.2863	0.246	0.53
	23	34.876	285.4	17.5	0.2486	0.794	0.746
50	1	2.896	2620.6	182.9	0.2152	5.24	0.66
	2	4.386	6424.8	656.3	0.1302	12.846	2.07
	3	4.577	499	112.2	0.0739	0.998	0.708
	4	4.786	7189.1	837.3	0.1442	14.374	0.734
	5	5.159	26068.1	1795.2	0.2114	52.122	0.55
	6	5.785	4402.5	1134.1	0.0593	8.802	1.011
	7	5.904	552.9	173	0.0533	1.105	0.648
	8	6.232	136.8	26.2	0.0849	0.273	2.121
	9	6.368	543.4	64.5	0.1262	1.086	0.439
	10	7.313	21.3	2.7	0.1248	0.043	0.536
	11	8.555	174.6	13.2	0.2048	0.349	0.825
	12	12.517	41.5	2.2	0.2994	0.083	0.86
	13	16.435	846.8	26.4	0.4875	1.693	1.031
	14	25.755	127	5.4	0.3622	0.254	1.041
	15	27.175	15.4	1.2	0.2076	0.031	0.887
	16	30.454	41.3	2	0.2861	0.083	0.978
	17	33.915	58.6	3.2	0.2663	0.117	0.607
	18	34.88	250.6	15.8	0.2426	0.501	0.783
34-50 Negative Control X	1	6.246	343.9	31	0.1855	22.672	2.926
	2	6.468	179.7	15.2	0.2018	11.85	0.606
	3	7.184	194.7	16.5	0.1817	12.837	0.909
	4	12.532	56.6	2.9	0.3145	3.731	0.881

	5	16.439	659.8	20.2	0.4943	43.501	0.992
	6	27.177	26.7	1.5	0.2593	1.761	1.506
	7	30.456	55.3	3.1	0.2522	3.649	0.925
34-50 Negative Control Y	1	6.245	321.5	29.5	0.1511	18.131	3.222
	2	6.471	202.2	16.6	0.2065	11.407	0.718
	3	7.19	106.8	8.8	0.1831	6.025	0.951
	4	12.551	95.4	3.4	0.403	5.379	1.294
	5	16.453	970.4	29.9	0.4984	54.73	0.989
	6	27.176	34.3	1.9	0.2648	1.936	1.548
	7	30.455	42.4	2.2	0.2664	2.391	1.056

Appendix 8. Results of TTX standard run on HPLC/UV/Vis spectrometer

PPM	Time	Area	Height	Width	Area %	Symmetry
Blank	22.689	36.3	2.4	0.2243	0.695	4.135
	23.325	1806.8	55.2	0.4201	34.594	0.748
	24.453	5.2	2.2	0.0398	0.099	1.369
	24.932	2368.6	225.1	0.1368	45.352	5.865
	25.015	443.2	115.3	0.0568	8.486	0.338
	25.203	342.6	41.4	0.1047	6.56	0.131
	26.509	11.6	2.7	0.067	0.223	0.688
	26.755	25.7	2.9	0.1573	0.493	0.995
	27.238	130.1	7.1	0.2402	2.49	0.821
	31.479	28.9	1.4	0.2559	0.553	2.442
	35.205	23.8	1	0.2746	0.455	1.003

Blank		0
	0.1	0
	0.1	0
	0.1	0
	0.5	0
	0.5	0
	0.5	0
	1	0
	1	0
	1	0

2	4.765	22.6	1.4	0.2079	100	0.381
2	4.777	21.4	1.4	0.2086	100	0.458
2	4.771	22	1.4	0.2077	100	0.433
4	4.673	47.4	2	0.3005	100	0.289
4	4.681	47.9	2.1	0.3008	100	0.318
4	4.715	46.8	2.1	0.2675	100	0.46
5	4.651	44.5	2	0.3633	100	0.521
5	4.645	40.1	1.9	0.3504	100	0.355
5	4.67	58.6	2.3	0.3092	100	0.398
10	4.493	19.3	1.1	0.2095	37.011	2.116
	4.785	32.8	2.4	0.2052	62.989	0.486
10	4.465	19.1	1.2	0.1976	37.53	1.456
	4.786	31.8	2.4	0.213	62.47	0.502
10	4.475	19.3	1.2	0.2004	37.06	1.857
	4.774	32.7	2.4	0.2068	62.94	0.476
50	4.739	77.6	3.1	0.4204	64.763	0.377
50	4.695	79.8	3.2	0.3228	100	0.283
50	3.953	31.5	1.8	0.215	21.369	0.754
	4.519	115.8	2.9	0.4771	78.631	0.178
100	3.267	10.4	1.1	0.1542	10.232	0.97
	3.73	24.9	1.3	0.2311	24.394	2.313
	4.651	66.6	2.4	0.4623	65.373	0.245
100	5.149	117.2	7	0.2243	100	0.966
100	5.165	123.3	6.9	0.2277	100	1.09

Appendix 9. Sequences obtained from genetic analysis performed on newts from Todmorden, England

N1

GCAGTACGGTTGAGGTATAACCCCAAATTGCCTCACGGCATATGCCGTGTTTGTGGGGACAAAGCC
CCATCGCGACAACACCCTCACTTATAAAGGGAGTCGAAGAATCCCCTGCCCTCTCCGCAATACCACCC
TATATATATCTCCAAAGCTCGCTACGTCATAAGTTATAAAAAAGGACGTCTTAGCCTCAACGCTCACC
CCCATAAATTCTTATTTGTGCGCCACATTCTCATTGGTGGGGCTCGGAGGACTCTAACCACCAACGAC
ACCCTTATCAGGGGTGCGCTCTATTAACCTGAGCAACAGAACCAAAGTCGTTTTTCTCGAATGGGGG
GCCCTGTCTGGATCTCAAAGACAACACCCTGTGTGCGTAGAAGGTGTTCTCCCACCTGAGATGAAGT
GCCCTGATGGGAGTTTCGCATATAGACCTGTGCCGATACGCATCTCTGAATGCCGGTAATTTGTGAG

N2

AGGATTTCACCCCAGATTGCCTAACCAGCAATTACGACGAATTCCAACTTCATGAAGTCAATTTGCAA
ATCCCCCCCCGAACTGAAATAGGGTTTCTGGAATTGCCTGACCCCCCCGGGTTGGCACCCCTCTGTCCC
TACCATTGCTTACTTGGGTAGCCCTGTTGAAAGGACCAGGATGACTTGACGTCCTCCCCCCCTTCCTC
GGGTTTGTCGCCCGCGCTCTCCTTAGTGTTCCCTCCATTAAGTGCTGGAACCTAGGGCGGAGGGTTG
CGGTGGGTGCGGAACTAACCCCCACGTCTCAAGACGAGATCTGAC

N3

TAGCCGACACTAGGCTACGGACAGAGATTTAACTCCTGCTGATGCTTTGAAGGCTTTGGACTTGGTA
TCCTAAGTCTTTAGTTTAGTACTGTTGGGGTAATTGGTATTATTAGCGCAGATGCCGTAAGTAACGAT
GCCGAGGTCCTGGAATCCAATTTCAAACTCCAATCCGGAATGATATAGGGTTTCCTGGATTGGCTTG
CCCTCCCCGGTTTGCAACCCTCTATCCCTACCCTTGGACTACGTGTGTAACCCTGGTAGTAAAGGACC
AGAAAGACTTGACGCATCCCCCCCTTCCTCCGGTTTGGCCCCGGCACTTCTCTTACAGTGTG

N4

TGAGACGGAGGGTTTGACCAAGGCCGGGGAAGGAATTCACGGCACAATGGCTAACCGGCAATTAC
AACGAATTCCAACTCCAGGAAGCCAATTTGCAAATCCCATCCCGAACGGAAATAGGGTTTCTGGAAT
TGCCTGACCCCCCCGGGTTGGCACCCCTCTGCCCCTACCATTGAATAACGTGGGTAGCCCTGTTCGA
AAGGACCATGATGACTTGACGTCATCCCCCCCTCCCTCCGGTTTGCCACCGGCGGTCTCCTTAAAGTT
CCCACCATTACTTGCTGGCACCTAAGGACAAGGGTTGCCCTCGTTGCGGAACTAAACCCAACATCAC
AAGACACGAGCTGACAACCGCCATGCACCACCTGTCTCACGGTTCCGAAAGGCACCACTCCATCTCT
GGAAAGTTCCGTGAATGTCAAGACCAGGTAAGGTTTTTC

N5

CCTTTGAAGGTCTTTGGCCTTGTTATCCTAAGTCTTTAGTTTAGTAGTGTTGGGGTAATTGGTATTATT
ATTGCGGATAATGTAATTAAAATTGGAATGGTAAAAGTTAATTTTTTTTCCCCCCGTCAAGTAAGATT
GGAGTTTGATATGTTTGGTGGGGAGGCAACACGTTTAAATAAATATCTCAAACTCTCGCTACGTCAC

AAGTTATAAAAGAACATGTCTCACCTCCACGCTTAGCCGTATAAATTCTTATGTGTGCACTACATTCA
GAGTGGTGGGTCTGGAATGACTCAAACCACCGACCTCACCCTTATCAGGGGTGCGCTCTAACCACCT
GACCTACACACCCAAAGTCTTTTCACTCAAATGGTGGAGCCTGTCGGGATCGAACCGACAACCCCCT
GCTTGCAAAGCATGTGCTCTCCCAGCTGAGCTAAGGCCCCATGATGAGACTTTCACATACCGGCCTG
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GA

N7

TCATTCTCGCCTGTGACCGGCTAGGCATCCACCGTGTGCGCTTATTCGCTTGACGGGGAACCCCAAG
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A

N8

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N9

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N10

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N11

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N12

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N13

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