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Development of a non-lethal biopsy technique for estimating total tetrodotoxin concentrations in the grey side-gilled sea slug *Pleurobranchaea maculata*

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ABSTRACT

High concentrations of tetrodotoxin (TTX) have been detected in some New Zealand populations of *Pleurobranchaea maculata* (grey side-gilled sea slug). Within toxic populations there is significant variability in TTX concentrations among individuals, with up to 60-fold differences measured. This variability has led to challenges when conducting controlled laboratory experiments. The current method for assessing TTX concentrations within *P. maculata* is lethal, thus multiple individuals must be harvested at each sampling point to produce statistically meaningful data. In this study a method was developed for taking approximately 200 mg tissue biopsies using a TemnoEvolution® 18G × 11 cm Biopsy Needle inserted transversely into the foot. Correlation between the TTX concentrations in the biopsy sample and total TTX levels and in individual tissues were assessed. Six *P. maculata* were biopsied twice (nine days apart) and each individual was frozen immediately following the second sampling. Tetrodotoxin concentrations in biopsy samples and in the gonad, stomach, mantle and the remaining combined tissues and fluids were measured using liquid chromatography-mass spectrometry. Based on the proportional weight of the organs/tissues a total TTX concentration for each individual was calculated. There were strong correlations between biopsy TTX concentrations and the total ($r^2 = 0.88$), stomach ($r^2 = 0.92$) and gonad ($r^2 = 0.83$) TTX concentrations. This technique will enable more robust laboratory studies to be undertaken, thereby assisting in understanding TTX kinetics, ecological function and origin within *P. maculata*.

1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin that functions by binding and obstructing voltage-gated sodium channels in nerve cell membranes, preventing the propagation of action potentials (Lu et al., 2011). Tetrodotoxin derives its name from the pufferfish family *Tetraodontidae* where it was first discovered, but it is now known to occur in a wide variety of phylogenetically distinct marine and terrestrial organisms (Ito et al., 2006; Hanifin, 2010; Williams, 2010). Despite many decades of research on TTX, its origins are still controversial with researchers either postulating an endogenous or exogenous source. Evidence for endogenous production comes from research on terrestrial organisms such as newts. Studies on captive newts (*Taricha granulosa*) found that TTX concentrations remained stable or increased over one year when fed TTX-free diets (Hanifin

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et al., 2002). It has also been demonstrated that TTX was released from the skin of *T. granulosa* when stimulated by a mild electric current, but concentrations in the skin increased again over nine months despite only being fed TTX-free earthworms and crickets (Cardall et al., 2004). Although no TTX-producing bacteria have been detected from terrestrial organisms (Lehman et al., 2004), they have been cultured from multiple marine organisms. However, the low TTX concentrations produced by bacteria in concert with non-specific and cross-reactivity problems with techniques used to measure TTX-production in bacterial strains have resulted in uncertainty regarding a microbial origin of TTX (Matsumura, 1995, 2001).

Between July and September 2009, there were a series of dog poisonings on beaches in Auckland, New Zealand. Investigations identified TTX in the dogs' stomachs and vomit, and in beach-cast *Pleurobranchaea maculata* (grey side-gilled sea slug). It was suggested that the dogs had ingested TTX from *P. maculata* or their toxic egg masses that had washed up on the beaches (McNabb et al., 2010). *Pleurobranchaea maculata* are opportunistic scavengers that are commonly found in shallow subtidal areas around New Zealand, and have also been recorded in south-eastern Australia, China, Sri Lanka and Japan (Willan, 1983). Research on TTX in *P. maculata* has found that populations in Auckland, Whangarei and Tauranga (Upper North Island, New Zealand) contain high concentrations of TTX ($>$1400 mg kg^{-1}) while populations in Wellington (Lower North Island, New Zealand) have low concentrations (*c.* 2.0 mg kg^{-1}) and South Island populations have no or very low concentrations ($<$0.01 mg kg^{-1}) of TTX (McNabb et al., 2010; Wood et al., 2012b). Wood et al. (2012b) showed that there was less than 1% sequence variability in the cytochrome *c* oxidase subunit 1 gene between the populations, providing evidence that they were the same species. In addition to between-population variability there are significant disparities in TTX concentrations within toxic *P. maculata* populations, with up to 60-fold differences occurring among individuals collected from one Auckland site (Wood et al., 2012b). Seasonal differences in TTX concentrations were also identified with a peak in June–July coinciding with egg-laying season (Wood et al., 2012b). A recent aquarium-based TTX-depuration study on *P. maculata* identified a similar trend when adults were maintained for 126 days on a TTX-free diet (Wood et al., 2012a). One of the limitations encountered during this study was the requirement to harvest multiple individuals at each sampling date due to the high natural variability in TTX concentrations and the need for lethal sampling to determine TTX concentrations (Wood et al., 2012a). For example, TTX concentrations measured in stomach tissue samples from Day 0 ranged from 14 to 1905 mg kg^{-1} among three individuals, making meaningful interpretation of changes in TTX concentrations challenging (Wood et al., 2012a). Development of a non-lethal technique for assessing TTX concentrations in *P. maculata*, allowing repeated assessment of one individual over time, would greatly assist future studies.

A non-lethal sampling method for assessing TTX concentrations in *T. granulosa* has been developed (Hanifin et al., 2004). They used a 5 mm diameter human skin biopsy punch to sample a region of the dorsal skin and developed a predictive model to estimate total TTX concentrations within the skin. Molluscs, however, present the further complication that their haemolymph ('blood') system lacks clotting factors; wounds may therefore haemorrhage uncontrollably, ultimately resulting in death (Armstrong et al., 1971; Hodgson, 1981; Taylor et al., 1994). Whilst maintaining *P. maculata* in aquaria we observed that when multiple individuals were kept together in one aquarium they often attacked each other removing significant portions of foot tissue. This did not always result in death of the injured individual (Wood and McNabb, unpub. data). The foot region consists of muscle and connective tissue and therefore represents a promising site for biopsy, as sampling this area would minimise the chance of lethal damage to internal organs. In terrestrial gastropods 'foot clipping' has been used previously to obtain tissue samples in order to prevent the need for lethal methods. For example, sampling of endangered Hawaiian tree snails (Achatinellinae) has been achieved using a sterile scalpel to obtain a 1–2 mm tissue slice from their foot region with no post-procedure mortality observed (Thacker and Hadfield, 2000).

The aims of this study were to develop a non-lethal biopsy method for sub-sampling *P. maculata* and to determine if the TTX concentrations in these sub-samples could be used to estimate total TTX concentrations in the entire organism, or among specific organs/tissues.

2. Methods

2.1. Field sampling and laboratory conditions

Nine *P. maculata* were collected by divers (18 July 2012) from Pilot Bay (Tauranga, New Zealand; 37°38′07″S, 176°10′29″E). Each was placed in a separate plastic bag containing seawater (300 mL) and transported back to the laboratory in an insulated container. *Pleurobranchaea maculata* were maintained in separate aquaria (19 L) filled with 14 L of aerated 0.22 μm-filtered seawater. At 2–3 day intervals *P. maculata* were fed Greenshell™ mussel (*Perna canaliculus*) sourced from the Marlborough Sounds (South Island, New Zealand) and their water was exchanged. To confirm that the mussels contained no TTX three from this batch were tested for TTX as described below.

2.2. Biopsies

Six of the nine *P. maculata* were biopsied (1 August 2012) while the remaining three were used as unmanipulated control samples. *Pleurobranchaea maculata* were removed from aquaria and transferred to beakers containing *c.* 300 mL seawater and allowed to acclimate for five minutes. Individuals were transferred to a damp paper towel on a bench top and held firmly with one hand. Biopsies (*c.* 200 mg) were taken using a TemnoEvolution® 18G × 11 cm Biopsy Needle inserted transversely into the foot (Fig. 1), dorsal to the pedal sinus and anterior to the pedal gland. Resulting tissue samples were placed into 1.7 mL tubes (Axygen). Control *P. maculata* were subject to the conditions above and were prodded with a metal rod to

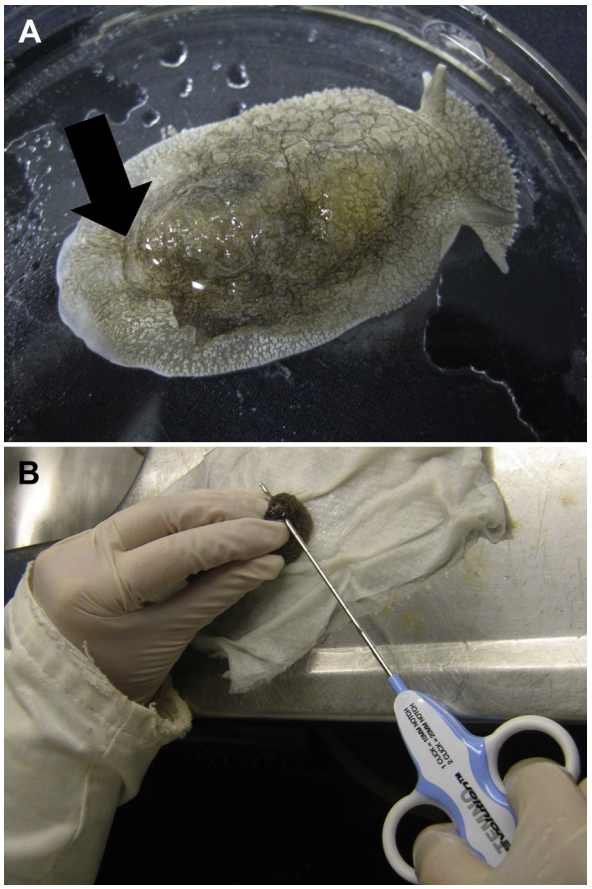

Fig. 1. (A) *Pleurobranchaea maculata*, arrow shows approximate site of biopsy. (B) Biopsies were taken for the foot of *Pleurobranchaea maculata* using a TemnoEvolution® 18G × 11 cm Biopsy Needle.

simulate the handling stress of the biopsy procedure, without breaking the epidermis. *Pleurobranchaea maculata* were returned to their separate aquaria immediately after biopsies were taken.

Pleurobranchaea maculata were maintained in aquaria for nine days. During this time they were provided food on three occasions and monitored for signs of ill health (reduced activity and feeding; compromised adhesion). A second biopsy sample was taken (10 August 2012) using the method described above but with the exception that the beakers contained no seawater to allow a mucus sample to be collected. All *P. maculata* in the study were frozen (−20 °C) whole immediately after biopsies were taken and a sample of mucus (*c.* 100 μL) collected from the beaker and frozen (−20 °C).

2.3. Dissection, TTX extraction, and analysis

Pleurobranchaea maculata were partially defrosted and dissected using a sterile scalpel. The gonad, stomach including gut contents, and a section of the mantle were removed. The gonad is attached directly to the stomach and due to difficulties separating these organs, emphasis was placed on taking a clean sample rather than on trying to remove the entire gonad. All remaining fluids and tissue were combined, homogenized, and labelled as the 'rest'. All sub-samples were weighed and frozen (−20 °C) for later TTX analysis.

Sub-samples (*c.* 1 g) of each organ/tissue were extracted with 9 mL of Milli-Q water containing 0.1% v/v acetic acid, or a *pro rata* volume if the starting mass was less than 1 g. Each sample was homogenized (1 min; Heidolph Diax 600 Homogeniser, Heidolph, Germany). Samples were centrifuged (3000 × *g*, 10 min) and an aliquot of the supernatant (100 μL) added to 900 μL of 100% methanol containing 0.1% v/v acetic acid and frozen (−20 °C) for at least 1 h. Samples were centrifuged (3000 × *g*, 10 min) and diluted 1:4 with 100% methanol containing 0.1% v/v acetic acid. The mucus samples (100 μL) were defrosted and extracted with 900 μL Milli-Q water containing 0.1% v/v acetic acid. These were vortexed and sonicated (15 min) and then processed as described above. Samples were analysed for TTX using liquid chromatography–mass spectrometry (LC–MS) as described in McNabb et al. (2010). Total TTX concentrations for each *P. maculata* were calculated using the TTX concentration and the proportional weights of the gonad, stomach, mantle, and 'rest'.

Mann–Whitney *U* tests were undertaken in Statistica 8 (StatSoft Inc.) to compare mean TTX concentrations in the gonads, stomach, mantle and 'rest' among biopsied and control *P. maculata*. Sequential biopsy results were compared by paired *t*-test; as this procedure identified no significant difference, paired biopsies from each individual were subsequently treated as duplicates and an average calculated. Linear regression was used to test the relationship between average TTX concentrations in the biopsy, and total and individual organ/tissues.

3. Results

Immediately after the first biopsy two out of six *P. maculata* experienced some bleeding from the site of the needle insertion. One of these individuals displayed inhibited mobility for approximately two hours post-biopsy. No other signs of behavioural change or inhibited mobility were observed during the remaining nine days. The control and biopsied *P. maculata* showed no decline in their considerable appetite for Greenshell™ mussel flesh. All individuals ate two to three pieces (c. 0.5 g) of mussel flesh on the three occasions when they were fed post biopsy.

Tetrodotoxin was the only variant detected in the samples. The TTX concentrations in the biopsy samples varied from 8 to 167 mg kg wet tissue^{-1} (Table 1). In general there were only minor differences in TTX concentrations between the first and second biopsy samples (Table 1). The exceptions were the biopsies from the third individual in which the TTX concentration in the first biopsy was three times higher than the second, and the fifth individual where the second biopsy sample was 2.7 times higher than the first (Table 1). No consistent difference was detected between paired samples ($t_{0.025,\ 5\ df} = -0.126$), sequential biopsies were therefore treated as replicates.

There were strong correlations between total ($F_{1,5} = 30.44$, $p = 0.005$, $r^2 = 0.88$), stomach ($F_{1,5} = 46.55$, $p = 0.002$, $r^2 = 0.92$) and gonad ($F_{1,5} = 19.48$, $p = 0.012$,

Table 1
Weights of, and tetrodotoxin (TTX) concentrations in biopsy and mucus samples taken from six *Pleurobranchaea maculata*.

No.	TTX (mg kg wet tissue^{-1})					
	#1	#2	#3	#4	#5	#6
P. maculata weight (g)	16.4	7.9	7.2	19.8	13.3	20.0
Biopsy 1	13	19	81	144	62	61
Biopsy 2 (+9 days)	8	31	26	117	167	48
Difference ratio between biopsy samples	1.6	0.6	3.1	1.2	0.4	1.3
Mucus	Trace	Trace	Trace	1.2	Trace	1.1

$r^2 = 0.83$) TTX concentrations and TTX concentrations in the biopsy samples (Fig. 2a and b). The correlations were lower for the mantle ($F_{1,5} = 3.80$, $p = 0.12$, $r^2 = 0.49$), and the 'rest' ($F_{1,5} = 7.88$, $p = 0.05$, $r^2 = 0.66$). Only trace levels of TTX were detected in four of the six mucus samples. The mucus samples from the two remaining *P. maculata* (the fourth and sixth) contained 1.2 mg kg^{-1} and 1.1 mg kg^{-1} respectively.

The average TTX concentrations in each organ/tissue were generally higher in the biopsied specimens than the control group (Fig. 3). These differences, however, were not significant; stomach ($Z = 1.549$, $N = 6,3$, $p > 0.5$), gonad ($Z = 1.549$, $p > 0.05$), mantle ($Z = 1.29$, $p > 0.05$), and 'rest' ($Z = 1.29$, $p > 0.05$). There was greater variability in TTX concentrations in the biopsy group (Fig. 3). For example, stomach TTX concentrations ranged between 14 and 285 mg kg^{-1} in the biopsied *P. maculata*, whereas the range in concentrations was only 23–25 mg kg^{-1} in the control group.

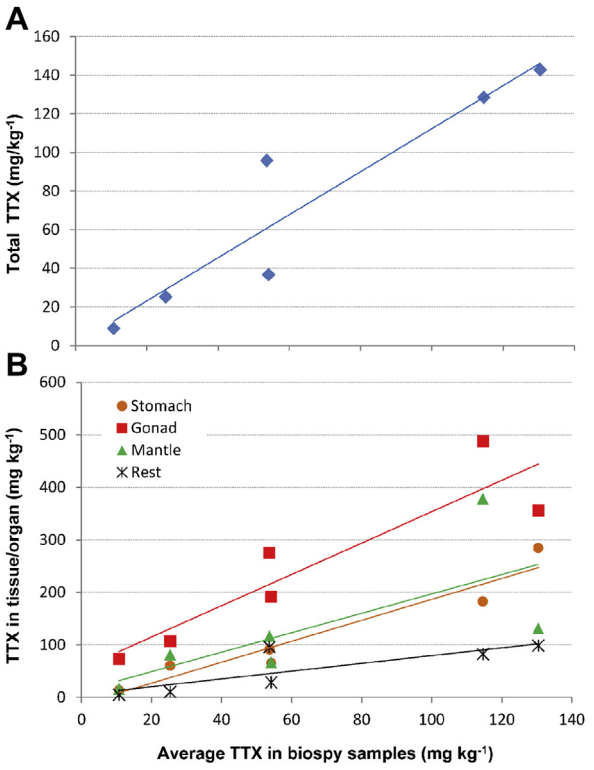

Fig. 2. (A) Total tetrodotoxin (TTX) concentration in six *Pleurobranchaea maculata* plotted against average TTX concentrations from two biopsy samples (c. 200 mg) taken nine days apart. (B) TTX concentrations in the stomach, gonad, mantle and 'rest' of six *P. maculata* plotted against average TTX concentrations from two biopsy samples.

4. Discussion

A range of non-lethal methods have been developed for sampling organisms in response to a need to investigate biological and physiological patterns, or to track contaminates within species and populations through time without causing long-term harm to individuals. Collecting representative samples from vertebrates is usually relatively straightforward and often involves collecting feathers, hairs, faeces, urine or taking skin samples; all of which can be undertaken with minimal disturbance to the individual (Beja-Pereira et al., 2009). Invasive sampling can also readily be undertaken on larger vertebrates, for example, biopsy techniques are routinely used in studies on marine mammals where non-lethal sampling techniques enable the acquisition of fresh skin and blubber samples from free-ranging animals (Noren and Mocklin, 2012). Non-lethal sampling of invertebrates, especially small organisms, is more challenging. A variety of techniques have been developed and the type of method used is largely dependent on the downstream application of the sample. For example, a simple swab of foot tissue mucus has been shown to provide enough material for genetic analysis (Palmer et al., 2008), but this technique is unlikely to be sensitive enough to track concentrations of contaminates in individuals. This was illustrated in the current study where although trace concentrations of TTX were detected in the mucus samples, they did not correspond to the actual TTX concentrations in individuals. If tissue is sampled in molluscs, particular care is required to avoid damaging the main vascular spaces which, in the absence of clotting mechanisms, is likely to cause uncontrolled bleeding (Armstrong et al., 1971; Hodgson, 1981; Taylor et al., 1994). In bivalves several research groups have developed non-destructive biopsy methods which either involve removing small sections of the mantle (Berg et al., 1995; Buhay et al., 2002; Grobler et al., 2006; Kochzius and Nuryanto, 2008) or ligament (Doherty et al., 2007). There are far fewer studies exploring non-lethal sampling in gastropod molluscs. The effects of foot clipping on mortality and behaviour of terrestrial Cumberland Tigersnail (*Anguispira cumberlandiana*) was investigated in Tennessee, USA, with no mortality reported after a 2-week period, although short-term behavioural changes were observed (Haskell and Pan, 2010).

In this study, a non-lethal biopsy technique for sampling tissue from the marine opisthobranch *P. maculata* was developed. Short-term behavioural changes were observed in one *P. maculata*, but these lasted less than two hours and no mortality was observed in any of the study organisms during the study period. It is likely that this technique could be applied to other Heterobranchia and the samples could be used for a range of applications. Measuring the TTX concentration in the biopsy sample also proved to be a robust method for estimating the total TTX concentrations within various tissues of adult *P. maculata*. The TTX

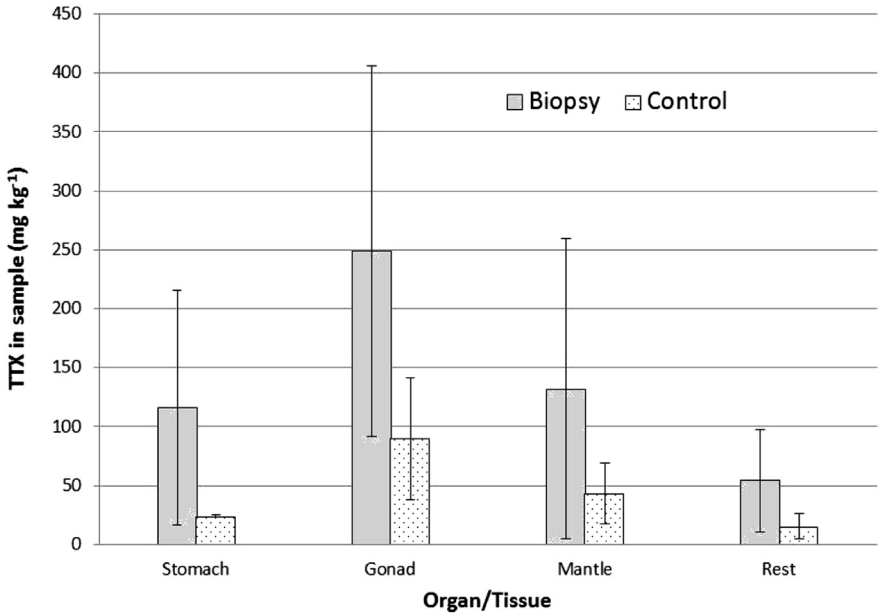

Fig. 3. Tetrodotoxin concentrations in organs/tissue of biopsied ($n = 6$) and control ($n = 3$) *Pleurobranchaea maculata*. Error bars show one standard deviation.

concentrations in the small (*c.* 200 mg) tissue biopsy samples had a strong correlation ($r^2 = 0.88$) with the total TTX concentrations. We had anticipated that we would need to develop a predictive model, similar to that of Hanifin et al. (2004) that took into account the difference in TTX concentration within various organs and their proportional weights; however, because of the strong correlation between the biopsy samples and total concentrations this was not necessary. Surprisingly, the lowest correlation was obtained when the biopsy TTX concentrations were compared to the mantle samples, which were taken in close proximity to each other. The biopsy sample was taken from within the foot rather than from the surface and this may explain why the biopsy sample is more predictive of total TTX concentrations rather than the surface concentrations. There were no significant differences in TTX concentrations between the biopsied and control *P. maculata*, indicating that the procedure had no effect on TTX concentrations within individuals.

The goal of the present study was to assess how much variability was present between biopsy samples taken within a short period and how these values related to TTX concentrations within specific organs and *P. maculata* as a whole. Wood et al. (2012a) showed marked decreases in TTX concentrations over three week sampling periods when *P. maculata* were maintained in captivity. To ensure that TTX concentrations did not decline relative to the biopsy samples, individuals in our current study were frozen immediately after conducting the second biopsy. This has limited our knowledge on potential long-term effects of the biopsies and how frequently biopsies can be taken. Wound closure in gastropods relies upon localised muscular contraction to occlude the lesion while a temporary plug of haemocytes forms; subsequent tissue regeneration typically requires 2–3 months (Armstrong et al., 1971; Taylor et al., 1994). In addition to its other vascular functions, haemolymph in the foot region serves a hydraulic function, antagonising muscle contraction, allowing extension and locomotion (Voltzow, 1986). A biopsy wound in the foot therefore incurs the additional risks, beyond haemorrhage and infection, that locomotion and adhesion could be impaired. However, given that there were no observed effects during the study and previous observation of long-term survival after conspecific attack (Wood, pers. obs.), we suggest that long-term effects would be minimal. A current feeding study is underway to assess the uptake of toxins into *P. maculata* and to date taking biopsies at a frequency of one every three weeks has not resulted in mortalities (Wood & McNabb, unpub. data).

Hanifin et al. (2004) used a 5 mm diameter human skin biopsy punch to sample several regions of skin from newts (*T. granulosa*) and determined TTX concentrations. The predictive model they developed has been utilized for multiple studies including: monitoring change in TTX concentrations during experimentation (Cardall et al., 2004), feeding studies (Williams et al., 2004, 2010) and geographic distribution surveys (Hanifin et al., 2008). It is anticipated that the biopsy technique developed in this study will be used for similar studies on *P. maculata*.

The high variability in TTX concentrations between individual *P. maculata* measured in this study was similar to those observed previously (Wood et al., 2012a, 2012b). Marked spatial and temporal variability in TTX concentrations has been recorded for many TTX-containing organisms including; *T. granulosa* (Hanifin et al., 1999), the horseshoe crab *Carcinoscorpius rotundicauda* (Dao et al., 2009), and gastropods (*Rapana rapiformis* and *R. venosa venosa*) (Hwang et al., 1991). Variables that lead to the striking differences in TTX content in these organisms are unknown, but the results from the present study and Wood et al. (2012b)

demonstrate that there is no correlation with the size or weight of an individual. In this study, variability amongst organs within individual *P. maculata* was shown with the gonads always containing the highest TTX concentrations. Wood et al. (2012a) undertook at 126-day captive study in which TTX-containing *P. maculata* were fed a TTX-free diet. Most organs depurated TTX, whereas there was only weak evidence for depuration of the gonad, indicating active transport of TTX to this organ. The ability to actively transport TTX may partially explain the variability observed within *P. maculata*. We speculate the individuals used in this study were close to egg laying, which would account for the high TTX-concentrations measured in the gonad.

In summary, the biopsy method developed by the present study provides a non-lethal method for sampling *P. maculata*. The aim of this study was to use biopsied samples to estimate TTX concentrations within individuals, but these samples could equally be used to monitor other physiological responses. For example, changes in catecholamines, or to obtain samples for genetic analysis in *P. maculata*, and potentially other Heterobranchia. There was a strong correlation between the TTX concentration measured in the biopsy samples and the total TTX in each *P. maculata*. This method will minimise the number of *P. maculata* that need to be sacrificed during population studies and assist in future captivity experiments. This technique will enable TTX concentrations within individuals to be tracked over many months and will ultimately assist in understanding TTX kinetics, function and possibly the origin in *P. maculata*.

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Conflict of interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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