Chemical cue interactions between alien invasive and native aquatic gastropods in the iSimangaliso Wetland Park, South Africa.

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As the candidate's Supervisor, I have approved this thesis for submission.

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Abstract

The importance of chemical cues in mediating interactions among individuals and structuring communities is being increasingly recognized in aquatic environments. Chemical cues have been shown to drive predator-prey interactions in which behavioural responses in terms of movement have been reported. The role of chemical cues in mediating interactions between heterospecific competitors, in which there is an observed behavioural response, has not been previously investigated. This research project has used the biological invasion of Tarebia granifera, a caenogastropod endemic to south-east Asia, to determine the role of chemical cues in driving displacement interactions with native gastropods within coastal lakes and estuarine environments of the iSimangaliso Wetland Park, in northern KwaZulu-Natal, South Africa. An experimental approach was used to measure behavioural responses of gastropods to chemical cue treatments by quantifying components of movement. Responses of three dominant native gastropods within Lake St Lucia were tested in a preliminary in situ experiment. The mean Displacement, mean Number of Steps and grand mean Turning Angle were determined from recorded pathways of individual gastropods using image-processing software. Responses to chemical cues released by T. granifera were significantly different and negative in comparison to the control and conspecific chemical cue treatments. Following these results, the experiment was repeated and refined to include responses between native heterospecific gastropods from populations within the larger geographical range of the iSimangaliso Wetland Park. Additionally, the case of whether the observed responses could be attributed to build up of metabolic waste products was also investigated. The results confirmed those of the preliminary *in situ* study. Negative responses were only reported for gastropods which did not have a shared evolutionary history with T. granifera, irrespective of metabolic waste concentrations Native gastropods moved away from chemical cues released by T. granifera using a directed orientation mechanism. Interestingly, native

i

gastropods did not exhibit this behavioural response to chemical cues of other heterospecifics. This suggests that *T. granifera* has developed chemical cues under evolutionary pressure, which act as deterrent on naïve gastropods. The implications for these responses are considered within the framework of Movement Ecology with contributions to Chemical Ecology. The potential of this interaction as a mechanism of invasion to be included within spread models is discussed.

Preface

The following MSc thesis was undertaken at the School of Life Sciences, University of KwaZulu-Natal, Westville. The work described was carried out from November 2012 to August 2013 under the supervision of Prof. R Perissinotto and Dr NAF Miranda.

The work presented in this thesis has not been submitted previously for any degree or diploma to any other tertiary institution. The studies are the original work of the author and where the work of others has been used, the sources have been acknowledged in the text.

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Plagiarism Declaration

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Publication Declaration

The following details the contribution to the publication which forms part of the research presented in this thesis as Chapter 1.

Raw JL, Miranda NAF, Perissinotto R (2013) Chemical cues released by an alien invasive aquatic gastropod drive its invasion success. *PLoS ONE* 8, e64071.

Author contributions: Conception and design: Raw, Miranda, Perissinotto. Performed experiments: Raw. Analyzed data: Raw, Miranda. Wrote the draft paper: Raw

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vi

Table of Contents

General Introduction1
Chapter 1: Chemical cues released by an alien invasive gastropod drive its
Invasion success
Chapter 2: Movement ecology: responses to chemical cue interactions between
alien invasive and native aquatic gastropods11
Introduction11
Materials and Methods12
Results20
Discussion
General Discussion and Conclusion including recommendations for future
work
Literature Cited

General Introduction

General Introduction

The systematic study of biological invasions began with the work of Elton (1958) which recognized the potential impacts of introduced species on biodiversity. Invasion Ecology has since developed into a multi-disciplinary field, which is continuously advancing with new key technologies and the emergence of new themes and research questions (Richardson and Pyšek 2008). Studies have typically focussed on dispersal and spread of non-native introduced species, as well as the roles of disturbance and enemy release on successful establishment (Lockwood et al. 2007). More recently, biological interactions between individuals have been increasingly recognized as important factors which influence invasion success (Simberloff and Von Holle 1999, Bulleri et al. 2008).

Behavioural traits which are induced by interactions between introduced and native species have been considered intrinsically important for determining the successful establishment and spread of the non-native species, or the resistance of the native species (see Dick 2008, Cote et al. 2010 and Chapple et al. 2012). Furthermore, behavioural responses of individuals are generally measured in terms of movement, which can be linked to dispersal and population processes through the framework of Movement Ecology (Hawkes 2009). In this manner, changes in behaviour at the level of individuals have the potential to culminate into changes at higher ecological levels.

Interactions that induce behavioural responses are not limited to physical interactions, especially in aquatic environments where the role of chemical cues has been widely documented as driving behavioural responses of native prey species to introduced non-native predator species (Pizzatto and Shine 2009, Freeman et al. 2013). However, the importance of chemical cues as drivers of interactions between heterospecific competitors has not been thoroughly investigated.

General Introduction

Chemical cues have been identified as having important functional roles in aquatic environments, as the interactions which they mediate can influence community structure (Hay 2009). The overwhelming significance of chemical cue interactions in aquatic environments is presumed to have developed under evolutionary pressure. Turbid benthic environments restrict the use of visual cues (Dodson et al. 1994), thus prompting aquatic organisms to utilize chemical cues for predator avoidance (Gutierrez et al. 2013), foraging (Fink et al. 2006) and habitat selection (Steinberg et al. 2002). Chemoreception is thus an important mechanism for receiving abiotic and biotic environmental information. Chemical cues with a deterrent or defensive function have also been identified from aquatic organisms, including macrophytes, phytoplankton and invertebrates (Sotka et al. 2009). Among the invertebrates, the evolutionary pressure to develop these substances is related to their vulnerability as soft-bodied sessile or slow-moving organisms (Amsler et al. 2001)

Gastropods have historically been the focus of chemoreception studies (Kohn 1961) and recent research has identified many responses to chemical cues corresponding to predators (Klose 2011) and food resources (Fink et al. 2006). Additionally, chemical substances with deterrence properties have been isolated from gastropods, with the focus on identifying compounds with biotechnological potential for clinical use (Benkendorff 2010). The aim of this research project was to determine whether chemical cues drive the competitive interaction between native gastropods and the alien invasive gastropod, *Tarebia granifera*, within the iSimangaliso Wetland Park. Results from stable isotope analysis by Miranda and Perissinotto (2012), showed that dietary overlap does exist between *T. granifera* and native gastropods in the region. However, there was no evidence for direct exploitative competition. Despite this, in environments where *T. granifera* has become established, the species dominates the benthos at the expense of native species which are reported to decline in

abundance (Miranda et al. 2011). Therefore the role of chemical cues in driving the competitive interaction was investigated.

Tarebia granifera is a parthenogenetic caenogastropod endemic to south-east Asia, which was first reported from South Africa in 1999 (Appleton and Nadasan 2002). *T. granifera* has a substantial tolerance for salinity, despite being considered a freshwater species (Miranda et al. 2010). This has allowed the species to successfully establish in coastal lakes and estuarine environments in northern KwaZulu-Natal (Appleton et al. 2009). These include areas within the iSimangaliso Wetland Park, a UNESCO World Heritage Site containing three Ramsar Wetlands of International Importance. *T. granifera* is currently successfully established in both Lake Sibaya and Lake Nhlange of Kosi Bay (Miranda et al. 2011). In Lake St Lucia its distribution was previously limited to environments with freshwater inputs from streams and aquifers on the Eastern Shores, while the system experienced hypersaline conditions (Miranda et al. 2010). However, following the reconnection of the Mfolozi River at the St Lucia Estuary Mouth and the increase in freshwater input due to rainfall in 2012, the system has generally tended towards a more oligohaline state. The distribution of *T. granifera* is expected to expand as environmental conditions become more favourable.

The spread of *Tarebia granifera* following introduction has been analysed through mechanistic models which predict rapid colonization across small distances (Facon and David 2006, Snider and Gilliam 2008). This research project measured the behavioural responses in terms of movement of native gastropods in response to chemical cues released by *T. granifera*. It was hypothesized that chemical cues drive a displacement interaction among heterospecific gastropods, which are competing for food and space resources. It was predicted that native gastropods would respond to chemical cues released by *T. granifera* by moving away from the source of these chemical cues.



Chemical Cues Released by an Alien Invasive Aquatic Gastropod Drive Its Invasion Success

4

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Abstract

Background: Chemical cues provide aquatic organisms with sensory information that guides behavioural responses and thus interactions among themselves, each other and the environment. Chemical cues are considered important for predator avoidance, foraging, larval settlement and broadcast spawning in aquatic environments. However, the significance of their role as drivers of direct interactions between heterospecifics has been largely overlooked.

Methodology/Principal Findings: A video camera and a demarcated arena were used *in situ* to record behavioural responses of three native gastropod species, *Assiminea* cf. *capensis, Melanoides tuberculata* and *Coriandria durbanensis*, exposed to treatments representing chemical cues released by a non-native invasive gastropod, *Tarebia granifera*. The responses were measured quantitatively as displacement and orientation of movement at locations in St Lucia Estuary, within the iSimangaliso Wetland Park, a UNESCO World Heritage Site on the east coast of South Africa. All native gastropods exhibited a negative taxis response to chemical cues released by *T. granifera*, while *T. granifera* individuals responded randomly to conspecifics. Displacement was measured relative to the source of the extract, the number of steps taken were determined with path analysis and orientation was determined from the mean (\pm 95% CIs) turning angles, with significant negative turning angles representing negative taxis. Responses to treatments corresponding to the environment and conspecifics were random and undirected, indicating kinesis.

Conclusion/Significance: This study presents evidence for interactions driven by chemical cues between a non-native invasive gastropod and several gastropods native to South Africa. The results indicate that chemical cues can facilitate invasion success as the behavioural response of native gastropods is to move away allowing additional food and space resources to become available to *T. granifera*.

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Introduction

Biological invasions, although considered a major component of anthropogenic global change, also provide an opportunity to study ecological principles which govern the coexistence of populations within communities [1]. Studies of biotic interactions such as predation, competition and facilitation between non-native and native species can translate to a better understanding of the relationships which exist among established native species [2]. Classic works considered exploitative competition as the primary biotic factor influencing the success of non-native species in new environments [3]. However, recent studies have emphasised interference competition and facilitation [4,5], as well as the importance of environmental factors that together may contribute towards invasion resistance [6].

Biotic interactions remain an important component of invasion success [2] and are generally investigated at the level of the individual by quantifying behavioural responses [7]. Research has focussed on the interactions between agonistic competitors [8] and novel predator-prey relationships [9]. These studies have quantified behavioural responses in terms of activity levels, aggression, dominance and searching for refuge. However, this classification has not accounted for behavioural responses of individuals prior to confrontation, or for species that are not naturally aggressive. In order to assess the other facets of this phenomenon, it is necessary to examine communication as a function of behavioural responses [10]. Various types of communication exist among organisms and thus both the concept and definition have been scientifically debated since the 1960s [11,12,13]. Despite the conceptual debate, chemical communication among organisms was recognized earlyon as an important component influencing behaviour [14,15].

The importance of chemical cues in structuring aquatic communities has been addressed extensively by empirical studies [16]. Responses in aquatic organisms have been demonstrated for chemical cues corresponding to natural predators [17], food sources [18] and conspecific alarm cues [19]. These methods have been applied to investigate biotic interactions between native and non-native species with emphasis on naïve predator-prey interactions [20,21,22]. This study provides empirical support for

chemical cues as a driver of important biotic interactions between native and non-native species.

Chemoreception has been comprehensively studied in gastropods [23] and many representatives of this class have become nonnative invasive species of global importance [24]. Considering these two factors, gastropods have become the focus of behavioural response studies involving interactions mediated by chemical cues. As chemoreception is known to guide locomotion [23], empirical studies have quantified behavioural responses in terms of movement [25]. The aim of this study was to determine whether chemical cues released by a non-native gastropod, *Tarebia* granifera, drive interactions with three native gastropod species, Assiminea cf. capensis, Melanoides tuberculata and Coriandria durbanensis, by affecting their behaviour and thus facilitating its invasion success.

Tarebia granifera, a freshwater gastropod endemic to south-east Asia, has invaded many tropical and sub-tropical parts of the world [26] and has most recently become established at the expense of native species in areas of the iSimangaliso Wetland Park and many other shallow water ecosystems in KwaZulu-Natal, South Africa [27]. Generally it has been assumed that *T. granifera* displaces native gastropods through exploitative competition. However, as food resources are not limiting in iSimangaliso [28], it was hypothesized that chemical cues are involved in displacement interactions between *T. granifera* and native gastropod species.

Materials and Methods

Ethics Statement

All necessary permits were obtained from the iSimangaliso Wetland Park Authority for the described field studies at each location, under a Research Agreement for the project titled "Climate Change and the Management of KZN Estuaries: St Lucia Estuary".

Gastropods and Experiment Sites

The behavioural responses of the three native gastropod species, Assiminea cf. capensis, Melanoides tuberculata and Coriandria durbanensis, to chemical cues released by *T. granifera* were quantified independently in terms of movement and orientation following a protocol adapted from Wollerman et al. [25]. The experiments for this study were carried out in March 2012 at Charter's Creek (28°12'3.29''S 32°25'6.26''E) and Catalina Bay (28°13'28.89''S 32°29'11.79''E) and in July 2012 at Lister's Point (27°58'22.30''S 32°22'28.24''E) and St Lucia Estuary Mouth (28°22'48.80''S 32°25'18.63''E) (Fig. 1) [29]. Salinity and temperature were measured with a YSI 6600-V2 multiprobe at each site.

Lake St Lucia, the largest estuarine lake in Africa [29], is located within the iSimangaliso Wetland Park, which is a UNESCO World Heritage Site. St Lucia has experienced low water levels following a decade of prolonged mouth closure [30] and diversion of the Mfolozi River away from the St Lucia Estuary. Consequently, a reverse salinity gradient has formed with the northernmost regions experiencing hypersaline conditions until very recently [31]. The South Lake region is substantially less saline than the northern reaches, as a result of freshwater seepage from aquifers along its eastern shores and inputs from rivers [31].

The non-native species, *Tarebia granifera*, despite being considered a freshwater caenogastropod, has a remarkable tolerance for moderately saline conditions of up to 30 [32] and has thus become successfully established in areas of the St Lucia Narrows (average salinity 13.8), as well as Catalina Bay (average salinity 3.6). *T.* granifera has however not yet been found at Charter's Creek



Figure 1. Locations of *in situ* experiments. Map of St Lucia Estuary, which is one of three Ramsar Wetlands of International Importance within the iSimangaliso Wetland Park in KwaZulu-Natal, South Africa (a UNESCO World Heritage Site since 1999). doi:10.1371/journal.pone.0064071.g001

(average salinity 4.0) on the western shore of the South Lake, while the salinity (54.4) at Lister's Point in False Bay is well beyond its physiological tolerance [32].

In the St Lucia Estuary, A. cf. capensis and C. durbanensis (previously collectively referred to as A. bifasciata or A. cf. ovata) have been reported to inhabit moderately saline to hypersaline environments with sandy substrata, where they are an important component of the benthos [33]. Experiments with A. cf. capensis (3– 5 mm shell height (SH)) were carried out at Catalina Bay where T. granifera (8–10 mm SH) currently dominates the benthic invertebrate assemblage, as well as at Charter's Creek where the nonnative species has either not been introduced or has not yet become established. Experiments with C. durbanensis (2–4 mm SH) were carried out at Lister's Point in the northern region of the system, where salinities are too high for T. granifera. Experiments

with *M. tuberculata* (8–10 mm SH), which is a widespread freshwater species in South Africa [34], were carried out at the St Lucia Estuary Mouth, where *T. granifera* has not become established, although it has been reported in the adjacent Narrows (Fig. 1). Experiments were conducted on the species present at each site at the time of the experiments. The only overlapping distributions were between *A.* cf. *capensis* and *T. granifera* at Catalina Bay.

Experiments and Data Analysis

Individual gastropods were exposed to chemical cue extracts that formed treatments of (1) conspecific cues, (2) cues released by the non-native species and (3) a control treatment representing general environmental cues at the time of the experiment. For sites where T. granifera does not occur, extracts were prepared with individuals collected from Catalina Bay and the salinity of the extracts was raised to that of the site prior to the experiment. Extracts were prepared in 2 L buckets with 300 ml of filtered water (Millex GP 0.2 µm) and gastropods collected from the respective site one day prior to the experiment. The gastropods were added to the buckets and kept for a fixed period to allow conditioning of the water with naturally-released chemical cues. Cue concentration was controlled for by conditioning the collected volume with the same biomass (wet weight) of gastropods for each treatment for a fixed period of time. Final densities were similar to those observed for each gastropod species in their respective habitats. During the conditioning period, gastropods were provided with naturally-occurring benthic algae collected in situ on which they fed ad libitum. The control treatment was prepared as above, but contained no gastropods. After conditioning, the water was once again filtered to remove any particulate organic wastes and accumulated microbes. A volume of 50 ml was retained and frozen for subsequent nutrient analysis while 8 drops of food colouring (Robertsons BLUE) was added to the remaining extract to aid monitoring of its delivery during the experiment.

Experiments were carried out in a tank $(90 \times 32 \times 38 \text{ cm})$ using small amounts of filtered water ($\pm 1 \text{ cm}$ depth) from the site. Gastropods were collected immediately prior to the experiment. An inverted video camera was used to record behavioural responses and a Perspex arena, with two concentric demarcated circles (diameters 10 and 20 cm), was used for reference. Extracts were continuously delivered to the centre of the arena at a rate of 1.5 ml/min by a titration burette mounted 5 cm above the centre of the arena. After two minutes of extract delivery, single individuals were placed on the edge of the inner circle, facing the source. The response was recorded for three minutes.

The response to each treatment was quantified by calculating the displacement (D) relative to the source of the extract for each individual snail. The displacement of a snail was calculated by subtracting the distance from the extract source to the endpoint of the pathway of the snail (E) from the distance from the source to the origin of the pathway of the snail (O). Thus, D is positive if the snail moved towards the source and negative if the snail moved away from the source. Differences between D for each treatment were tested with ANOVA. Differences between the numbers of steps taken by snails in each treatment (see below) were also tested using ANOVA. There were 5 species/locations (A. cf. capensis at Catalina Bay, A. cf. capensis at Charter's Creek, C. durbanensis at Lister's Point, M. tuberculata at St Lucia Estuary Mouth and T. granifera at Catalina Bay), 3 treatments (conspecific cue, non-native species cue and control) and 10 replicates per treatment. Out of the 150 individuals used in experiments, 5 were excluded from the dataset as they displayed unusual behaviour and/or impaired activity.

Path analysis was done according to the method of Benhaumou and Bovet [35]. The response pathway of the snails was reduced to a sequence of changes of direction, each with its own sidedependent turning angle (α) . The side-dependent turning angle is the angle between the direction of snail movement and the direction of the source of the extract. The turning angle is positive if the snail turns toward the stimulus. If the snail turns away from the stimulus, the turning angle is negative. Turning angles therefore fall within the range of -180° to 180° . If a snail orients using kinesis, it is equally likely to turn towards (positive α) or away (negative α) from the source after each step, regardless of the direction of its previous step. In this case, the mean of the distribution of turning angles (mean α) will not be significantly different from 0. However, if the snail orients using taxis, it will consistently turn towards the source (positive taxis) or away from the source (negative taxis) and the mean α will be significantly different from 0. First, the mean turning angle (mean α) of each individual snail was calculated. The mean turning angles for each treatment were then averaged to determine the grand mean turning angle (grand mean α). Finally, a one-sample t-test (twotailed) was used to compare each grand mean α to zero. To measure turning angles, the pathway of each snail was reduced to a number of discrete linear steps by reconstructing its pathway using a constant step length R [35]. Trials were conducted to determine an appropriate step length for discretizing snail pathways, using control treatments of each snail species. The path of each snail was discretized three times, with step lengths of 0.4, 0.8, and 1 cm. A mean α for each pathway was calculated using each step length, with a Kruskal-Wallis non-parametric analysis of variance showing no significantly different results between the three step lengths. A step length of 0.8 cm was chosen for all analyses because it produced a discretized pathway that was easy to measure and resulted in many turning angles. The number of steps taken by snails was determined according to the standardized step length and interpreted as a proxy for physical activity.

The pathways were traced, scanned and processed with ImageJ software. The 5 individual outliers contributed 6% of the path analysis data which were excluded from the analyses. All assumptions for parametric tests were satisfied. Data were analyzed and the "displacement" and "steps" graphs were generated in IBM SPSS v.19 for Windows. Turning angle graphs were generated with Corel Draw X5 for Windows.

Results

Responses were measured in terms of distribution of turning angles, displacement and number of steps taken (Figs. 2, 3, 4, 5, 6).

The mean turning angle of all native species was only significantly different from zero in response to the Tarebia granifera cue treatment: Assiminea cf. capensis at Catalina Bay (t=-4.894, d.f.=8, P=0.001) (Fig. 2); A. cf. capensis at Charter's Creek (t=-4.221, d.f.=9, P=0.002) (Fig. 3); Coriandria durbanensis at Lister's Point (t=-5.335, d.f.=5, P=0.003) (Fig. 4); and Melanoides tuberculata at the St Lucia Mouth (t=-4.664, d.f.=9, P=0.001) (Fig. 5). The mean turning angle of the non-native T. granifera (Fig. 6) was not significantly different from zero for any of the treatments (t-tests, d.f.=5, P>0.450). Mean turning angle was always negative for the T. granifera cue treatment (i.e. turned away from source, Figs. 2, 3, 4, 5), except when T. granifera was the test subject.

An analysis of variance of the mean displacement of gastropods produced a significant difference between treatments ($F_{2,145} = 5.533$, P = 0.031) (Table S1 in File S1). Tukey's HSD Post-



Figure 2. Assiminea cf. capensis orientation and movement responses to different chemical cue treatments at Catalina Bay. Three types of data are presented: (1) Mean turning angles distribution in response to chemical cue treatments (pie graphics). Arrows represent the overall mean for the treatment and shaded slices represent 95% CIs. Type of motion, i.e. taxis or kinesis, is indicated; (2) Mean displacement (\pm s.d.) of individuals relative to the source of chemical cue (bar graphics); (3) Average number of steps taken by individuals during the experiment (colour legend: yellow = 3 to 5 steps, orange = 6 to 9 steps, orcurred are demarcated. Individuals were tracked over a period of three minutes.

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hoc test revealed that the *T. granifera* extract treatment resulted in a significantly different (P < 0.05) and negative (i.e. away from the source) movement response by all of the native gastropod species (Tables S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12 in File S1), except *M. tuberculata* which displayed a negative but not significant ($F_{2, 29} = 2.488$, P = 0.102) movement response (Fig. 5, Table S7 in File S1). There were also no significant differences, in terms of displacement between all treatments for *T. granifera* ($F_{2, 29} = 3.334$, P = 0.051) (Table S5 in File S1).

An analysis of the mean number of steps taken by gastropods revealed no overall differences between treatments ($F_{2, 145} = 2.010$, P = 0.196) (Table S13 in File S1), except for A. cf. *capensis* at Charter's Creek ($F_{2, 29} = 5.763$, P = 0.008) where the mean number of steps was higher for the *T. granifera* extract treatment than for the others (Fig. 3, Tables S23 and S24 in File S1).

Discussion

Historically, chemoreception studies have emphasized the identification of the chemical compound that stimulated a behavioural or physiological response. Consequently, a large proportion of substances have been identified as stimulants including those which promote or deter feeding, promote metamorphosis, evoke predator avoidance, attract mates or influence habitat selection [36,37]. In contrast, the majority of studies that focus on interspecific interactions mediated by chemical cues have rarely identified the chemical structure of the cues themselves [20,25,38].



Figure 3. Assiminea cf. capensis orientation and movement responses to different chemical cue treatments at Charter's Creek. Three types of data are presented: refer to Fig. 2. Treatments where a negative (-ve) taxis response occurred are demarcated. Individuals were tracked over a period of three minutes. doi:10.1371/journal.pone.0064071.g003

Although this study has not identified, isolated and purified the chemical cue in question through biochemical assays, the significant quantified behavioural responses suggest that the



Figure 4. Coriandria durbanensis orientation and movement responses to different chemical cue treatments at Lister's **Point.** Three types of data are presented: **refer to Fig. 2**. Treatments where a negative (–ve) taxis response occurred are demarcated. Individuals were tracked over a period of three minutes. doi:10.1371/journal.pone.0064071.g004



Figure 5. *Melanoides tuberculata* orientation and movement responses to different chemical cue treatments at St Lucia **Estuary Mouth.** Three types of data are presented: **refer to Fig. 2**. Treatments where a negative (-ve) taxis response occurred are demarcated. Individuals were tracked over a period of three minutes. doi:10.1371/journal.pone.0064071.g005

interspecific interaction among Tarebia granifera and native gastropods is driven by chemical cues. Metabolic wastes generally cause avoidance responses [36]; however preliminary results from the inorganic nutrient analysis revealed no significant difference in ammonium levels between extracts. It is therefore unlikely that the extracts contained cues derived from high levels of nitrogenous wastes, which would cause an avoidance response in all gastropod species. The results of these and further nutrient analyses are part of a separate study which is being prepared for publication. A more probable explanation is that the avoidance responses are caused by a secondary metabolite, possibly a biogenic volatile organic compound (VOC), released either by T. granifera itself or by microbial epibionts. VOCs are acknowledged as important infochemicals, most notably for their role in mediating interactions between plants and herbivores in terrestrial systems, however their ecological significance in the aquatic environment has become more recently recognized [39]. VOCs have been reported to mediate interspecific interactions [40], where they function as kairomones with only the recipient benefitting from the interaction [41]. However, there is a need for further research on other interactions which are potentially mediated by VOCs in the aquatic environment [39].

The behavioural responses, quantified as three components of movement: mean displacement, mean number of steps taken and mean turning angle, illustrate how chemical cues released by the non-native *T. granifera* affect native gastropod species (Figs. 2, 3, 4, 5). All gastropods displayed kinesis to orient in response to the control and respective conspecific treatments (Figs. 2, 3, 4, 5, 6). This indicates that movement in response to general environmental cues (control treatment), as well as chemical cues released by conspecifics, is random and undirected [35]. *T. granifera* responded in a similar way to the source of chemical cues released by the native *Assiminea* cf. *capensis* (Fig. 6). However, all three native species (Figs. 2, 3, 4, 5), displayed negative taxis to orient their



Figure 6. *Tarebia granifera* orientation and movement responses to different chemical cue treatments at Catalina Bay. Three types of data are presented: **refer to Fig. 2**. Individuals were tracked over a period of three minutes. doi:10.1371/journal.pone.0064071.g006

movement in response to chemical cues released by the non-native T. granifera. This indicates that the movement of all three native species in response to chemical cues released by T. granifera was non-random [35] and in a direction away from the source. Remarkably, the same response was found in A. cf. capensis individuals from Charter's Creek, Coriandria durbanensis individuals from Lister's Point and Melanoides tuberculata from the St Lucia Mouth, none of which had any physical pre-exposure to T. granifera individuals.

Coriandria durbanensis individuals were generally more active than the other two native species (Fig. 4). This is unlikely to be due to the average temperature prevailing during the experiments, which was similar between sites for *C. durbanensis* at Lister's Point (21.3°C) and *A.* cf. capensis at Catalina Bay (22.2°C). The highest temperature recorded was in March at Charter's Creek (27.1°C) while the lowest was in July at the St Lucia Estuary Mouth (18.5°C). *A.* cf. capensis individuals from Catalina Bay (Fig. 2) took fewer steps on average and tended to move towards, and stay close to, the source, when exposed to the conspecific treatment, suggesting an aggregation response [16,42]. In contrast, *A.* cf. capensis individuals at Charter's Creek took more steps on average, and tended to move away and stay away from the source, in response to the *T. granifera* chemical cue treatment (Fig. 3).

Response to the control treatment in terms of displacement varied among species and was dependent on conditions at the site during the experiment. *T. granifera* individuals were observed to move closer to the source of chemical cues released by conspecifics and the native *A.* cf. *capensis*, which is currently still present at Catalina Bay (Fig. 6). Gastropods that co-habit areas have exhibited movement as a response to heterospecific alarm cues [43], which assists in predator avoidance. However, the response of *T. granifera* in this case could contribute towards a more directional range expansion towards areas already inhabited by native gastropods. These behavioural responses involving both orientation and movement away, in a non-random directed manner, from the source of a chemical cue released by the nonnative *T. granifera*, may have direct consequences for the spatial distribution of native gastropods.

The release of chemical cues can cause heterospecifics to move away from an area. This may explain how T. granifera displaces native species. However, since native hetrospecifics were not present in any of the sites during the course of this study, their effect could not be tested. Therefore, explicit conclusions cannot be made about the mechanism at work in interactions between non-native and native species unless a native hetrospecific treatment is included in the analyses. Our understanding of the biotic interactions among different species is still incomplete, especially concerning aquatic organisms. Further studies are needed to identify the biochemical properties of the chemical cue released by T. granifera, and possibly also by other heterospecifics, which lead to avoidance responses in gastropod species.

Supporting Information

File S1 Table S1. Results of ANOVA testing for differences in snail (n = 145) displacement between experimental treatments at St Lucia Estuary. Table S2. Results of Tukey HSD testing for differences in snail (n = 145) displacement among experimental treatments at St Lucia Estuary. Table S3. Results of ANOVA testing for differences in Assiminea cf. capensis (n = 29) displacement between experimental treatments at Catalina Bay. Table S4. Results of Tukey HSD testing for differences in Assiminea cf. capensis (n = 29) displacement among experimental treatments at Catalina Bay. Table S5. Results of ANOVA testing for differences in Tarebia granifera (n = 29) displacement between experimental treatments at Catalina Bay. Table S6. Results of Tukey HSD testing for differences in Tarebia granifera (n=29) displacement among experimental treatments at Catalina Bay. Table S7. Results of ANOVA testing for differences in Melanoides tuberculata (n = 29) displacement between experimental treatments at St Lucia Estuary Mouth. Table S8. Results of Tukey HSD testing for differences in Melanoides tuberculata (n = 29) displacement among experimental treatments at St Lucia Estuary Mouth. Table S9. Results of ANOVA testing for differences in Coriandria durbanensis (n = 29) displacement between experimental treatments at Lister's Point. Table S10. Results of Tukey HSD testing for differences in Coriandria durbanensis (n = 29) displacement among experimental treatments at Lister's Point. Table S11. Results of ANOVA testing for differences in Assiminea cf. capensis (n = 29) displacement between experimental treatments at Charter's Creek. Table S12. Results of Tukey HSD testing for differences in Assiminea cf. capensis

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Chemical Cues Drive Gastropod Invasion Success

(n = 29) displacement among experimental treatments at Charter's Creek. Table S13. Results of ANOVA testing for differences in average number of steps taken by snails (n = 145), between experimental treatments at St Lucia Estuary. Table S14. Results of Tukey HSD testing for differences in average number of steps taken by snails (n = 145), among experimental treatments at St Lucia Estuary. Table S15. Results of ANOVA testing for differences in average number of steps taken by Assiminea cf. *capensis* (n = 29), between experimental treatments at Catalina Bay. Table S16. Results of Tukey HSD testing for differences in average number of steps taken by Assiminea cf. capensis (n = 29), among experimental treatments at Catalina Bay. Table S17. Results of ANOVA testing for differences in average number of steps taken by *Tarebia granifera* (n = 29), between experimental treatments at Catalina Bay. Table S18. Results of Tukey HSD testing for differences in average number of steps taken by Tarebia granifera (n = 29), among experimental treatments at Catalina Bay. Table S19. Results of ANOVA testing for differences in average number of steps taken by *Melanoides tuberculata* (n = 29), between experimental treatments at St Lucia Estuary Mouth. Table S20. Results of Tukey HSD testing for differences in average number of steps taken by Melanoides tuberculata (n = 29), among experimental treatments at St Lucia Estuary Mouth. Table S21. Results of ANOVA testing for differences in average number of steps taken by Coriandria durbanensis (n = 29), between experimental treatments at Lister's Point. Table S22. Results of Tukey HSD testing for differences in average number of steps taken by Coriandria durbanensis (n = 29), among experimental treatments at Lister's Point. Table S23. Results of ANOVA testing for differences in average number of steps taken by Assiminea cf. capensis (n = 29), between experimental treatments at Charter's Creek. Table S24. Results of Tukey HSD testing for differences in average number of steps taken by Assiminea cf. capensis (n = 29), among experimental treatments at Charter's Creek.

(DOC)

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Author Contributions

Conceived and designed the experiments: NM RP. Performed the experiments: JLR. Analyzed the data: JLR NM. Contributed reagents/ materials/analysis tools: RP. Wrote the paper: JLR.

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Movement ecology: responses to chemical cue interactions between alien invasive and native aquatic gastropods

1 INTRODUCTION

2 Chemical communication in aquatic systems has been long recognized as having immense ecological and evolutionary importance for both benthic and pelagic organisms (Lampert et 3 al. 1994). Enhanced chemoreception for foraging, predator avoidance and mate recognition 4 has evolved under environmental pressure in many aquatic representatives spanning various 5 6 taxa (Brönmark and Hansson 2000). Unsurprisingly, this produces a potentially vast diversity 7 of chemical substances in aquatic environments, the majority of which have not been 8 biochemically isolated or specifically identified as having functional roles in ecological 9 interactions (Burks and Lodge 2002). As chemical cues mediate interactions between individuals, responses to novel stimuli are particularly interesting. These naïve interactions 10 11 can be observed through biological invasions, which provide an ideal platform to measure responses between species that have not co-existed over evolutionary timescales (Lockwood 12 13 et al. 2007).

Interactions driven by chemical cues are most often quantified in terms of induced 14 behavioural or morphological responses (see Freeman et al. 2013). Behavioural responses are 15 16 often compared and measured in terms of movement and thus should be assessed within the conceptual framework of "Movement Ecology" described by Nathan et al. (2008). This 17 framework describes the measured movement of an individual as the culmination of four 18 major components, namely: 1) the physiological state of the individual; 2) the ability of the 19 20 individual to move in different modes; 3) the ability of the individual to navigate; and 4) the effect of external factors, including aspects of the biotic and abiotic environment. 21

The movement of individual organisms ultimately influences their interactions with otherindividuals and the environment. This in turn relates not only to species distribution but also

community composition (Holyoak et al. 2008). As such, movement ecology has particular
important implications for research on the spread of non-native invasive species (see Snider
and Gilliam 2008). Considering these implications and the importance of chemical
communication in the aquatic environment, the aim of this study was to assess changes in the
small-scale movement of native gastropods in response to chemical cues released by the nonnative invasive *Tarebia granifera*.

30 *Tarebia granifera* has successfully established itself in coastal lakes and estuarine

31 environments in northern KwaZulu-Natal, South Africa at the expense of native gastropods

32 (Appleton et al. 2009, Miranda et al. 2011).

The role of chemical cues in the interaction between *T. granifera* and three native gastropods 33 was investigated with in situ experiments by Raw et al. (2013). To verify and further test the 34 35 strength of those reported interactions, the current research investigated the responses of a larger number of native gastropod species from a wider geographical range as well as other 36 alien invasive species to chemical cues released by T. granifera. Additionally, responses to 37 38 heterospecifics besides T. granifera were also compared. It was hypothesized that native gastropods would exhibit a negative behavioural response only when exposed to chemical 39 cues released by T. granifera. The observed behavioural responses measured were related to 40 small-scale movement and thus the potential impact of this interaction as a mechanism 41 42 enhancing invasion success was predicted.

43 MATERIALS AND METHODS

44 **Ethics statement**

All necessary permits were obtained from the iSimangaliso Wetland Park Authority for the
described *in situ* experiments at each location. This study was conducted under a Research
Agreement for the project titled "Climate Change and Management of KZN Estuaries: St
Lucia Estuary Phase 2". The described laboratory experiments were performed at the School
of Life Sciences of the University of KwaZulu-Natal.

50 Gastropods and experiment localities

51 Behavioural responses to chemical cue treatments were investigated in five native gastropod

52 species: Assiminea cf. capensis, Bulinus natalensis, Coriandria durbanensis, Melanoides

tuberculata and *Nassarius kraussianus*. The responses of three non-native gastropods, *Aplexa marmorata*, *Radix rubiginosa* and *Tarebia granifera* were also investigated.

In situ experiments were carried out with gastropods collected from sites at the time of the 55 experiment. Figure 1 shows localities of experiments within the major coastal lakes of 56 northern KwaZulu-Natal in the iSimangaliso Wetland Park, namely St Lucia, Sibaya and 57 Nhlange. Experiments at sites within Lake St Lucia include the four cases described in Raw 58 et al. (2013) carried out: in March 2012 at (1) Charter's Creek (28°12'3.29''S 32°25'6.26''E) 59 and (2) Catalina Bay (28°13'28.89''S 32°29'11.79''E) and in July 2012 at (3) Lister's Point 60 (27°58'22.30''S 32°22'28.24''E) and (4) St Lucia Estuary Mouth (28°22'48.80''S 61 32°25'18.63"E). Additional experiments were carried out in November 2012 at Catalina 62 Bay. Experiments at Lake Sibaya (27°23'49.28"'S 32°42'42.17"'E) were carried out in 63 December 2012 and at Lake Nhlange (26°57'37.46'' S 32°49'36.77''E) of the Kosi Bay 64 system in January 2013 (Fig. 1). Additional in situ experiments were carried out at one site 65

outside the Park in January 2013, at Amatikulu Aquarium Plants (29°4'16.44''S 31°
38'55.05''E), a tropical fish and aquatic plant aquaculture initiative at the mouth of the
Matikulu Estuary. A YSI 6600-V2 multiprobe was used to measure salinity and temperature
at each site at the time of the experiment.

70 Laboratory experiments were carried out to replicate the *in situ* experiments, including those 71 of Raw et al. (2013). Laboratory experiments also investigated responses between 72 heterospecific gastropods which were not collected from the same sites but are reported to 73 have overlapping distributions. Gastropods were transported to the laboratory in plastic 74 buckets containing water from the respective sites. In the laboratory, the water was replaced with filtered sea water and de-chlorinated tap water mixed to the salinity of the water from 75 the site from which the gastropods were collected. The buckets were aerated and kept at a 76 constant temperature of 21.5°C under a 12L:12D photoperiod for an acclimation period of 2-77 5 days prior to the experiments. The gastropods were fed benthic algae collected off the 78 79 substrate at the St Lucia Estuary Mouth, which were cultivated under the same laboratory conditions. 80



Figure 1. Locations of *in situ* experiments and sites from which gastropods were
collected for laboratory experiments within iSimangaliso. Map of iSimangaliso Wetland
Park in northern KwaZulu-Natal, South Africa's first UNESCO World Heritage Site. The
Mlalazi and Matikulu estuaries are approximately 111 km and 137 km south of the St Lucia
Estuary mouth respectively.

Chapter 2

The iSimangaliso Wetland Park hosts Lake St Lucia, the largest estuarine lake in Africa 86 (Taylor et al. 2006), as well as Lake Sibaya, a prehistorically estuarine lake that no longer has 87 a connection to the Indian Ocean (Wright et al. 2000) and the Kosi Bay estuarine lake system 88 consisting of four lakes. In iSimangaliso, Tarebia granifera has successfully established in 89 90 both Lake Sibaya and Lake Nhlange (Miranda et al. 2011). In St Lucia its distribution is currently limited to Catalina Bay and the Narrows region upstream from the Mouth. 91 92 Assiminea cf. capensis and Coriandria durbanensis have both previously been referred to as 93 A. bifasciata or A. cf. ovata, as individuals of both species are small in size (3-5 mm shell 94 height, SH, and 2-4 mm SH respectively). These species historically constituted a large biomass of the benthos in the St Lucia system (Boltt 1975). Only adult A. cf. capensis 95 individuals were used in experiments, as they can be separated from C. durbanensis by shell 96 shape characteristics. In situ experiments with A. cf. capensis were carried out at Catalina 97 Bay and Charter's Creek in the South Lake of St Lucia. Experiments with C. durbanensis 98 99 were carried out in the northern region, at Lister's Point in False Bay. 100 In situ experiments with Bulinus natalensis (10-12 mm SH) and Aplexa marmorata (8-10 mm SH) were carried out at Catalina Bay at the beginning of the 2012/2013 wet season. A. 101 marmorata is a pulmonate gastropod endemic to South America, which was first reported in 102 Durban, South Africa, in 1986 (Appleton et al. 1989). B. natalensis is a native planorbid 103 104 gastropod and other members of this genus, B. africanus and B. globosus, are intermediate 105 hosts of trematode parasites of the Schistosoma genus (Appleton and Madsen 2012). In situ experiments with B. natalensis (6-8 mm SH) were also carried out at Lake Sibaya. 106 107 Melanoides tuberculata is a predominantly parthenogenetic thiarid gastropod, considered endemic to Africa and Asia, with a cosmopolitan distribution throughout South Africa (De 108

109 Kock and Wolmarans 2009). In situ experiments with M. tuberculata (8-10 mm SH) were

carried out at the St Lucia Estuary Mouth (SLEM), as well as at Lake Nhlange in Kosi Bay
(KBLN). For *in situ* experiments at Amatikulu Aquarium Plants (AEAP), *M. tuberculata* (1012 mm SH) were collected from the gutters alongside the cement ponds housing the tropical
fish. For laboratory experiments, *M. tuberculata* (18-20 mm SH) were also collected from the
Mpophomeni Stream in the False Bay region of Lake St Lucia (SLMS) (27°57'7.17''S
32°22'37.21''E).

116 Nassarius kraussianus has a large geographic distribution along the southern African coast,

117 inhabiting shallow intertidal mud-banks of estuaries from Algoa Bay extending up to

118 KwaZulu-Natal and Mozambique (Branch et al. 2010). The species was collected for

laboratory experiments from mud banks at the mouth of the Mlalazi Estuary (28°57'59.78''S

120 31°45'37.83''E) in Mtunzini.

Radix rubiginosa is a pulmonate gastropod endemic to south-east Asia and, like many other
lymnaeids, the species can serve as an intermediate host to the liver fluke *Fasciola* sp. (Kaset
et al. 2010). *In situ* experiments with *R. rubiginosa* were carried out at Amatikulu Aquarium
Plants.

125 Experimental protocol and data analysis

Behavioural responses of gastropods to chemical cues were quantified independently by a
series of experiments amounting to a total of 593 replicates (*n*) across all sites, species and
treatments after removal of outliers.

129 Individual gastropods were exposed to treatments of chemical cues corresponding to: (1)

130 Conspecific cues. (2) Cues released by *T. granifera*. (3) A control treatment representing cues

131 present in the environment. (4) Cues released by other heterospecific gastropods. The *in situ*

treatments (1), (2) and (3) were replicated in the laboratory, while treatment (4) was only

133 carried out in the laboratory. For each species, the experiment was replicated ten times *in situ* with treatments (1), (2) and (3) (n = 30). However, in some cases, for logistical reasons, the 134 135 experiment could only be replicated five times per treatment in situ (n = 15). Additionally, nwas reduced in cases where gastropods did not respond to the stimulus of the experiment, 136 137 possibly because of poor health conditions or quiescence. In the laboratory, the in situ treatments and the heterospecific chemical cue treatments were replicated ten times. Fewer 138 responses were recorded in laboratory experiments in cases where gastropods were 139 compromised after transportation and stressed under laboratory conditions. 140 141 Extract treatments were prepared following the protocol of Raw et al. (2013) for both *in situ* and laboratory experiments. The dissolved metabolic wastes accumulated during the 142 preparation of the extracts can be responsible for avoidance responses (Hay, 2009). As such, 143

144 the concentration of ammonium (NH_4^+) , as a component of dissolved inorganic nitrogen

145 (DIN), was measured by continuous flow analysis at the CSIR (Council for Scientific and

Industrial Research) in Durban. The relationship between the concentration of ammonium in
extract treatments and the observed behavioural responses of gastropods to those treatments
was assessed using Spearman's Rank Correlation. These analyses indicated whether observed
behavioural responses were correlated to large concentrations of nitrogenous wastes.

The experiments described follow the protocol of Raw et al. (2013). Chemical cue extract was introduced at a constant rate of 1.5 ml/minute to the centre of a circular Perspex arena, from which it diffused in all directions. The extract was introduced for a period of 2 minutes before individual gastropods were placed on the periphery of the arena. Their responses were recorded using an inverted video camera for 3 minutes per individual replicate.

From the recorded videos, the pathways were traced, scanned and digitized using theprocessing software ImageJ. The movement responses of gastropods to chemical cues were

Chapter 2

measured using the methods of Wollerman et al. (2003) and quantified as mean 157 "Displacement", mean "Number of Steps" and a grand mean "Turning Angle" in response to 158 each treatment for each species. Displacement was measured as the difference in the distance 159 of the individual gastropod from the source of the stimulus at the beginning and at the end of 160 the experiment. The Number of Steps was determined using the method of Benhamou and 161 Bovet (1992), by calculating a standardized step size (0.8 cm) which was then used to re-162 discretize the path for each individual. Turning angles were measured from the re-discretized 163 paths using the method of Benhamou and Bovet (1992). Angles were measured for each step 164 in relation to the direction of the previous step and the direction of the source of the extract. 165 The grand mean Turning Angle for each treatment was calculated from the mean angles of 166 each replicate. Individual replicates represent the responses of individual gastropods. 167 ANOVA was used to check that there were no significant differences in responses between 168 batches, which consisted of experiments carried out at different times and at different 169 170 salinities and temperatures for the same species. 171 ANOVA with Tukey's Post Hoc test was used to compare: (1) The mean Displacement and mean Number of Steps between treatments and between in situ and laboratory experiments 172 for each species. (2) Differences between experiments with individuals of the same species 173 collected from different sites. (3) The grand mean Turning Angles for each species between 174 in situ and laboratory experiments. Data were pooled if there was no significant difference 175

between results obtained with *in situ* and laboratory experiments, otherwise the data wereanalyzed separately.

The grand mean Turning Angle for each species in response to each treatment was compared
to zero using a One-Sample *t*-test, to determine the orientation mechanism as described by
Benhamou and Bovet (1992). A significantly different grand mean Turning Angle was

181 considered to be non-random and directed either towards (positive taxis) or away (negative

taxis) from the source of the stimulus. The response was considered to be random (kinesis) if

the grand mean Turning Angle was not significantly different from zero.

- 184 Outliers that were removed did not exceed 10 % of the original data. All assumptions for
- 185 parametric tests were satisfied. All statistical tests and the graphs of Displacement were
- 186 generated with IBM SPSS v.21 for Windows. The graphs for Turning Angles were generated
- 187 with Corel Draw X5 for Windows.

188 **RESULTS**

189 The behavioural responses of gastropods in terms of movement were quantified as

- 190 distribution of Turning Angles, mean Displacement and mean Number of Steps taken for
- 191 each species (Figs 2-12). Correlations between behavioural responses and the concentration
- 192 of metabolic waste products are also reported.

193 Turning Angles

- 194 Grand mean Turning Angles were only significantly different from zero for the following
- 195 native species in response to the *Tarebia granifera* extract treatment: Assiminea cf. capensis

196 $(t_{24} = -4.120, p = 0.0001)$ (Fig. 2); Bulinus natalensis $(t_{25} = -3.072, p = 0.005)$ (Fig. 4);

197 *Coriandria durbanensis* ($t_5 = -5.335 p = 0.003$) (Fig. 5); *Melanoides tuberculata* (SLEM) (t_{15}

198 = -4.632 p = 0.0001) (Fig. 6), *M. tuberculata* (SLMS) ($t_9 = -4.179 p = 0.002$) (Fig. 7) and

199 *Nassarius kraussianus* ($t_7 = -3.102 p = 0.017$) (Fig. 10). All significantly different turning

200 angles were negative. For all gastropod species, the grand mean Turning Angles were not

significantly different from zero in response to the control, conspecific and other

heterospecific chemical treatments (Figs 2-12). Additionally, no significant differences in the
 grand mean turning angles between *in situ* and laboratory experiments were observed for any
 gastropod species tested.

205 **Displacement**

An overall ANOVA comparison of mean Displacement for all gastropods showed significant differences between treatments ($F_{3,593} = 18.896$, p = 0.0001), with Tukey's Post-Hoc test indicating differences between the *Tarebia granifera* treatment and the control, conspecific and heterospecific treatments (p = 0.0001). This trend was not constant for species-specific comparisons as it was only observed in the following cases. (1) In *Assiminea* cf. *capensis*

 $(F_{6,97} = 4.571, p = 0.0001)$ between the *T. granifera* treatment and the control treatment (p =211 0.049) (Fig. 2). (2) In Bulinus natalensis ($F_{5.99} = 3.367$, p = 0.008) between the T. granifera 212 treatment and the conspecific treatment (p = 0.020) and the Melanoides tuberculata treatment 213 (p = 0.027) (Fig. 4). (3) In *Coriandria durbanensis* $(F_{4,35} = 3.474, p = 0.019)$ between the *T*. 214 215 granifera treatment and the control treatment (p = 0.032) and the A. cf. capensis treatment (p= 0.015) (Fig. 5). (4) Finally, in *M. tuberculata* (SLMS) ($F_{3,39}$ = 3.757, p = 0.019) between 216 the T. granifera and conspecific treatments (p = 0.012) (Fig. 7). A significant difference in 217 mean Displacement between conspecific and native heterospecific treatments was only 218 reported for A. cf. *capensis* and the B. *natalensis* treatment (p = 0.022) (Fig. 2). 219 Significant differences in mean Displacement between experiments with conspecifics 220 collected from different sites were only reported for *Melanoides tuberculata* ($F_{3,141} = 10.319$, 221 p = 0.0001) (Figs 6-9). Differences occurred between experiments with snails collected from 222 Amatikulu (AEAP) and Lake Nhlange of Kosi Bay (KBLN) (p = 0.037), AEAP and St Lucia 223 224 Estuary Mouth (SLEM) (p = 0.032), KBLN and SLEM (p = 0.0001) and KBLN and St Lucia Mpophomeni Stream (SLMS) (p = 0.001), as indicated by Tukey's Post Hoc tests. 225 Additionally, significant differences in mean Displacement between *in situ* and laboratory 226

227 experiments were only reported for *M. tuberculata* (KBLN) Fig. 8) and *Radix rubiginosa*

228 (Fig 11) ($F_{1,25} = 14.874$, p = 0.001 and $F_{1,43} = 14.157$, p = 0.001 respectively).

229 Number of Steps

230	An overall ANOVA	comparison of the mean	Number of Steps by	gastropods was
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significantly different between treatments ($F_{3,593} = 4.693$, p = 0.003) with Tukey's Post Hoc

- analysis indicating significant differences between the *Tarebia granifera* treatment and the
- control treatment (p = 0.028) and the conspecific treatment (p = 0.002). However, this trend
- 234 was not observed for all species. Differences between treatments were only observed for

- between the *Melanoides tuberculata* and conspecific treatments (p = 0.001) (Fig. 5).
- 238 Significant differences in mean Number of Steps between experiments with conspecifics
- collected from different sites were only found for *M. tuberculata* ($F_{3,141} = 3.730$, p = 0.013)
- 240 (Figs 6-9). Tukey's Post Hoc test revealed a significant difference between KBLN and SLEM

241 (p = 0.015).

- 242 When comparing the mean Number of Steps between *in situ* and laboratory experiments,
- ANOVA and Tukey's Post Hoc tests indicated differences in the following cases. (1) In A.cf.
- 244 *capensis* ($F_{2,73} = 6.008$, p = 0.004) for experiments at Charter's Creek (p = 0.003). (2) In
- 245 Aplexa marmorata ($F_{1,27} = 5.162$, p = 0.032). (3) In Bulinus natalensis ($F_{2,72} = 6.687$, p = 0.032).
- 246 0.002) for experiments at Lake Sibaya (p = 0.014). (4) In *M. tuberculata* (KBLN) ($F_{1,25} =$
- 247 8.613, p = 0.007). (5) In *M. tuberculata* (SLEM) ($F_{1,49} = 14.682$, p = 0.0001). (6) In *T*.
- 248 granifera ($F_{3,82} = 19.502$, p = 0.0001) for experiments at Lake Nhlange (p = 0.0001).
- 249 Significant differences in the mean Number of Steps between *in situ* localities were only
- reported for *B. natalensis* between Lake Sibaya and Catalina Bay (p = 0.003) and for *T*.
- 251 granifera between Lake Nhlange and Catalina Bay (p = 0.0001) and Lake Sibaya (p = 0.0001)
- 252 0.0001)

253 Correlations with dissolved inorganic nitrogen

- The concentration of dissolved inorganic nitrogen (DIN) was not significantly different
 between sites for replicated treatments as indicated by MANOVA.
- 256 Spearman's Rank correlations revealed no significant linear relationships between the
- 257 concentration of ammonium and grand mean Turning Angles or the mean Displacement.

Significant correlations were detected for the mean Number of Steps with the concentration of Ammonium (rho = -0.0115, p = 0.033). However, these reported correlations likely arose as the Number of Steps data are skewed towards zero, with very few replicates recording a large number of steps (> 20) while the Displacement and Turning Angle data range from negative to positive values.



Figure 2. Assiminea cf. capensis orientation and movement responses to different

chemical cue treatments. Three components of the behavioural response are represented.(1)

265 Mean turning angle in response to chemical cue treatments (pie graphics); arrows represent

the overall mean for the treatment and shaded slices represent 95% CIs; orientation

267 mechanism, i.e. taxis or kinesis is indicated.(2) Mean displacement (±1 SE) of individuals

- relative to the source of the chemical cue (bar graphics).(3) Mean number of steps taken by
- individuals during the experiment (colour legend: green = 4 to 6 steps, yellow = 7 to 9 steps,
- orange = 10 to 13 steps, red = 14 to 17 steps and violet = 18 to 24 steps). Data were pooled
- for *in situ* and laboratory experiments.



- 272 Figure 3. Aplexa marmorata orientation and movement responses to different chemical
- 273 cue treatments. Three types of data are presented: refer to Fig. 2. Data were pooled for *in*
- *situ* and laboratory experiments.



- 275 Figure 4. *Bulinus natalensis* orientation and movement responses to different chemical
- 276 cue treatments. Three types of data are presented: refer to Fig. 2. Data were pooled for *in*
- *situ* and laboratory experiments.



278 Figure 5. Coriandria durbanensis orientation and movement responses to different

chemical cue treatments. Three types of data are presented: refer to Fig. 2. Data were

²⁸⁰ pooled for *in situ* and laboratory experiments.



- Figure 6. *Melanoides tuberculata* from the St Lucia Estuary Mouth (SLEM), orientation
- and movement responses to different chemical cue treatments. Three types of data are
- presented: refer to Fig. 2. Data were pooled for *in situ* and laboratory experiments.



- Figure 7. Melanoides tuberculata from the St Lucia Mphophomeni Stream (SLMS),
- orientation and movement responses to different chemical cue treatments. Three types of
- 286 data are presented: refer to Fig. 2. Data represent laboratory experiments only.



287 Figure 8. *Melanoides tuberculata* from Kosi Bay Lake Nhlange (KBLN), orientation and

- 288 movement responses to different chemical cue treatments. Three types of data are
- presented: **refer to Fig. 2**. Data for *in situ* and laboratory experiments were analyzed
- separately.



- 291 Figure 9. Melanoides tuberculata from Amatikulu Estuary Aquarium Plants (AEAP),
- 292 orientation and movement responses to different chemical cue treatments. Three types of
- data are presented: **refer to Fig. 2**. Data were pooled for *in situ* and laboratory experiments.



- 294 Figure 10. *Nassarius kraussianus* orientation and movement responses to different
- chemical cue treatments. Three types of data are presented: refer to Fig. 2. Data represent
- 296 laboratory experiments only.



297 Figure 11. *Radix rubiginosa* orientation and movement responses to different chemical

- 298 cue treatments. Three types of data are presented: refer to Fig. 2. Data for *in situ* and
- 299 laboratory experiments were analyzed separately.



300 Figure 12. *Tarebia granifera* orientation and movement responses to different chemical

301 **cue treatments.** Three types of data are presented: **refer to Fig. 2**. Data were pooled for *in*

302 *situ* and laboratory experiments.

303 **DISCUSSION**

304

305 in different phases, which have specific proximate and ultimate drivers (Nathan et al. 2008). A variety of indices have been developed to assess certain characteristics of movement paths, 306 307 each with different sensitivities to sample size, scale and location errors (Almeida et al. 308 2010). Considering these factors, an index which can be applied universally to all studies does not exist, as the appropriateness of the index depends on the objectives of each study 309 (Almeida et al. 2010). This study has utilized components of the movement index described 310 311 as 'Sinuosity' by Benhamou (2004). These components, namely Displacement, Number of Steps and Turning Angles, were selected to quantify movement responses and determine 312 whether individuals oriented differently in response to a range of chemical cue stimuli. 313 314 The grand mean Turning Angle gives an indication of the orientation mechanism used by individuals in response to the chemical cue stimulus (Benhamou and Bovet 1992). In 315 response to the Tarebia granifera treatment, orientation by negative taxis was observed for 316 317 all native species (Figs 2, 4, 5, 6, 7, 10) with the exception of Melanoides tuberculata (KBLN) (Fig. 8) and (AEAP) (Fig. 9). The non-random orientation directed the movement of 318 native gastropods away from the source of chemical cues as indicated by the negative 319 Displacement relative to the source (Figs 2, 4, 5, 6, 7, 10). As reported by Raw et al. (2013) 320 this response was recorded for individuals that had not previously been exposed to T. 321 granifera. These included Assiminea cf. capensis from Charter's Creek, Coriandria 322 durbanensis from Lister's Point and M. tuberculata (SLEM). The same result was found in 323 the current study for *M. tuberculata* (SLMS) (Fig. 7) and *Nassarius kraussianus* (Fig. 10). 324 Interestingly, none of these gastropods exhibited a negative taxis response to the chemical 325 cue treatments of heterospecific gastropods, which were not collected from the same site. It is 326

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The conceptual framework of movement ecology requires that movement paths are described

evident that the observed behaviour is not simply a response to a chemical cue not
encountered during the life history of the individuals, but rather to a chemical cue specifically
associated with *T. granifera*.

The mean Number of Steps taken by gastropods is interpreted as a proxy for physical activity 330 levels. Aplexa marmorata (Fig. 2) and Nassarius kraussianus (Fig. 10) showed the highest 331 332 levels of physical activity. Physids, such as A. marmorata, have been shown to exhibit more rapid and directional motion in comparison to other gastropods (Appleton 2003), while rapid 333 movement in N. kraussianus has been attributed to the structure of the foot (Trueman and 334 335 Hodgson 1990). On average, the lowest levels of physical activity were recorded for Assiminea cf. capensis, Melanoides tuberculata (SLEM) and Tarebia granifera. Responses in 336 terms of activity to different chemical cue treatments varied between species with 337 pronounced differences observed only for A. cf. capensis and C. durbanensis. These 338 variations could be due to confounding factors, such as the life history of individuals 339 340 including the presence of predators in the environment and the state of quiescence at the time of collection. 341

Overall responses to treatments were not significantly different between *in situ* and laboratory 342 experiments for most gastropod species in terms of mean Displacement and grand mean 343 Turning Angle, despite differences in the mean Number of Steps. This indicates lower 344 activity levels in laboratory experiments, which could be attributed to stress from 345 346 transportation or laboratory captivity conditions. However, even at lower activity levels, individuals responded to treatments with the same mechanism of orientation as reported from 347 in situ experiments, illustrating a uniform response to particular treatments irrespective of 348 349 activity levels.

Chapter 2

The role of chemical cues in influencing behavioural responses in aquatic environments has 350 been overwhelmingly addressed through predator-prey interactions (see Schoeppner and 351 352 Relyea (2009), Peacor (2006) and Dalesman et al. (2006)). Investigations considering the role of chemical cues in competitive interactions are in comparison non-existent. This imbalance 353 is unexpected as the profound importance of chemical cues in mediating biotic interactions 354 and structuring aquatic communities is increasingly recognized (Hay 2009, Sotka et al. 2009). 355 This study has presented novel evidence for the role of chemical cues in mediating 356 interactions between heterospecific competitors. The small-scale changes in displacement 357 influenced by these interactions illustrate the role of chemical cues in influencing behaviour 358 and movement at the level of individuals. 359

As no correlations were observed between concentrations of nitrogenous wastes to the 360 quantified responses of gastropods, the possibility of nitrogenous waste causing the 361 avoidance response can be eliminated. High concentrations of ammonia are known to be 362 363 toxic in aquatic environments however, below certain thresholds it has been demonstrated 364 that the substance does not inhibit growth in *Biomphalaria glabrata* (Thomas et al. 1976). In 365 the extract preparations with Tarebia granifera the mean (±sd) ammonia concentration measured was $21.28 \pm 15.42 \,\mu\text{M}$ (unpublished data) which was not significantly different to 366 that of the other gastropod species. Filtration through the 0.2 µm filter ensured the removal of 367 all biological material and thus there was no accumulation of ammonia in the extract. The 368 369 possibility of the quantified behaviour being in response to a specific chemical cue associated with Tarebia granifera must therefore be considered. 370

The evolutionary significance of this chemical cue produced by *Tarebia granifera* needs to be addressed from the perspective of invasion biology. Early work by Prentice (1983) and Perez et al. (1991) found that *T. granifera* outcompeted *Biomphalaria glabrata*, an

intermediate host of Schistosoma mansoni and T. granifera has subsequently been used as a 374 biological control agent in the Caribbean (Pointier and McCullough 1989, Pointier and 375 Guyard 1992). Laboratory studies by Gomez et al (1990) confirmed competition between T. 376 granifera and B. glabrata over time despite non-limiting food resources and thus suggested 377 378 the role of a chemically mediated interaction. Evidence from the current study suggests that T. granifera has developed a chemical cue with distinct effects on the behaviour of gastropod 379 species, which are distantly related and do not share an evolutionary history with T. 380 granifera. Interestingly, for Melanoides tuberculata (KBLN) (Fig. 8) and (AEAP) (Fig. 9) the 381 reported behavioural responses were not different from T. granifera. M. tuberculata has a 382 cosmopolitan distribution with morphs that are endemic to Africa as well as south-east Asia 383 (Genner et al. 2004). It is currently unknown whether Asian M. tuberculata are present in 384 South African populations, as has been reported for Lake Malawi (Genner et al. 2004). 385 386 However, the variation in behavioural responses in *M. tuberculata* could thus be attributed as an artefact of having a shared geographic origin and interaction over evolutionary time with 387 T. granifera. 388

The evolution of a unique chemical cue in *Tarebia granifera*, which causes an avoidance 389 390 response in naïve competitors, would be a beneficial trait that enhances establishment success by reducing biotic resistance and thus could be characterized as a continuous disturbance. 391 This would in turn support "invasional meltdown" (Simberloff and Von Holle 1999) if the 392 presence of T. granifera facilitates the invasion of other non-native species. In the current 393 study, the invasive species Aplexa marmorata was also collected from Catalina Bay where T. 394 395 granifera dominates the benthos. A. marmorata did not have a negative response to the T. granifera treatment despite having different geographic origins. The random response of A. 396 397 marmorata is likely an artefact of the rapid movement exhibited by this species and not a true 398 representation of the invasional meltdown theory. Additionally, A. marmorata has not been

found to persist at Catalina Bay which suggests the species is more susceptible to changingenvironmental conditions than *T. granifera*.

401 Future work should determine the biochemical nature of the cue responsible for the observed behavioural responses. Additionally, the ability of native gastropods to become tolerant to 402 403 cues released by T. granifera after prolonged exposure in the environment should also be 404 considered. The case of Melanoides tuberculata should be further investigated, as the species 405 is closely related to T. granifera and the population at Lake Nhlange has shown some resistance to the non-native invasive species. The role of chemical cues in driving interactions 406 407 in freshwater environments presents a large gap in research in comparison to what is known about chemical ecology in the marine environment. It is likely that further investigation will 408 reveal a range of undiscovered compounds mediating many interactions, with implications 409 for explaining observed patterns in higher level organization such as species distribution. 410

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424 interests. Correspondence should be addressed to J.L.R (jackie.raw33@gmail.com).

General Discussion and Conclusion with recommendations for future work.

Only a small percentage of the chemical cues responsible for interactions have been isolated and identified at a biochemical structural level, mainly due to technical and logistical constraints (Burks and Lodge 2002). The vast majority are secondary metabolites (SM) produced by a wide range of organisms ranging from microbes to invertebrates (Sotka et al. 2009). By definition, SM do not have a role in primary internal metabolism (Fraenkel 1959) and as such must have evolved under selective pressure as a defensive, protective or communicative mechanism for soft-bodied sessile or sedentary organisms. In the case of Tarebia granifera, if the chemical cue is in fact a secondary metabolite, it is unknown whether it evolved under pressure from predation or from competition. Of the SM identified for Gastropoda, the largest proportion are classified generally as terpenes which characteristically have deterrence properties (Benkendorff 2010). Research has focussed on marine species because of the potential biotechnological developments into natural products for clinical use. However, according to Benkendorff (2010), the absence of an acquired immune system in the Mollusca emphasizes the need to produce and respond to SM as defence and escape mechanisms, respectively. These attributes are shared by marine and freshwater species, thus suggesting that a large number of SM are yet to be discovered from freshwater species, particularly from the Gastropoda as it is the most diverse class of the phylum.

This research project has used the introduction of an alien invasive gastropod, *Tarebia granifera*, to examine the role of chemical cues in driving competitive interactions with native gastropod species within the iSimangaliso Wetland Park, South Africa. The preliminary study found that native gastropods exhibited a distinct avoidance response to chemical cues released by *T. granifera*. Further investigations confirmed these results and

found that in contrast, gastropods which shared an evolutionary history or geographic origin with *T. granifera* responded randomly to these chemical cues. The hypothesis is supported, as chemical cues released by *T. granifera* have been shown to cause avoidance responses in native gastropods.

The reported displacement interaction between *Tarebia granifera* and native gastropods adds to the list of traits which contribute the profound success of this invasive species. Wide physiological tolerances to ranges of salinity and temperature as reported by Miranda et al. (2010) have allowed this species to establish in a variety of freshwater and estuarine environments ranging from rivers to coastal lakes. Additionally, the parthenogenetic reproductive strategy of *T. granifera* essentially ensures continuous propagule pressure and theoretically a new population can be founded by one individual (Miranda et al. 2011). Furthermore, it has been reported that the species is spread to new locations by surviving being ingested and passed through the digestive system of waterbirds (Appleton et al. 2009). These traits that enable translocation to, and survivability and persistence within new habitats are complemented by the chemical interaction that *T. granifera* has with native gastropods. Once a population is established, the response of native gastropods to the chemical cue is likely to make more food and space resources available to *T. granifera* and thus possibly induce a positive feedback. This has the potential to have large consequences if it occurs in every new area into which the species is introduced.

Future studies should address the biochemical isolation and identification of chemical cues released by *T. granifera*. The minimum concentration at which the cue is effective on native species could then be determined. This would give an indication of whether there is a threshold population size of *T. granifera* at which this chemical cue interaction influences invasion success. Studies on the persistence of the chemical cue in the aquatic environment

will determine the scale at which this interaction can impact native gastropod species. The nature of the cue can be inferred from the observations of this study. Firstly, only adult *T. granifera* individuals were used to prepare the extract treatments and the avoidance response of native gastropods was the same irrespective of locality or season. This indicates that the chemical cue is continuously released by adult *T. granifera* irrespective of immediate environmental conditions or seasonal variations. Secondly, the extract treatments were prepared over a period of ten hours before use in experiments. This indicates that the chemical cue is relatively stable in the aquatic medium, which suggests it may be transported within the environment and still be viable. Thirdly, in cases where the salinity *in situ* was beyond the physiological tolerance for *T. granifera*, as reported for experiments with *Coriandria durbanensis* at Lister's Point, the extract was raised to the measured salinity after preparation. As a significant response was still recorded for *C. durbanensis*, this indicates that the chemical cue remains viable at higher salinities and was still effective after dilution.

The physiological source of the suspected chemical cue has not yet been identified, however early studies on other gastropod species provide some insight to likely exudates. The natural regulation of gastropod population density has been attributed not only to metabolic waste build up (Thomas et al. 1976) but also to the presence of natural inhibitors which may also limit growth (Levy et al. 1973). These natural inhibitors have been investigated by Berrie and Visser (1963) for *Biomphalaria sudanica* in Uganda where a dissolved organic compound which limited growth in natural populations was isolated and identified. Thomas et al. (1975) and Levy et al. (1973) report the presence of growth-inhibiting substances in the faeces of both *B. glabrata* and *Fossaria cubensis* respectively. However, a study by Madsen (1984) found that water conditioned with *Helisoma duryi* did not have an inhibitory effect on *B. camerunensis* adults or juveniles. Either *H. duryi* does not produce an inhibitory substance or the regular removal of faeces from experimental cages did not allow the build up of a

threshold concentration. The potential of the chemical cue released by *Tarebia granifera* being present in the faeces should be investigated. Under the experimental conditions, native gastropods were exposed to these cues directly and at a concentration that would be higher than in the natural environment. As such, responses were swift and in a non-random direction. However, in the natural environment chemical substances would likely be broken down or removed by currents. The patchy distribution of native gastropods that are found in areas where *T. granifera* is well established should be investigated as a factor which is influenced by small scale variations in water movement between microhabitats and the population density of the invasive species.

The quantification of gastropod movement undertaken in this study allows for further research on this phenomenon to be carried out within the framework of movement ecology (Nathan et al. 2008). The application of existing movement indices (Almeida et al. 2010) would allow for pathways of individual gastropods to be analysed as a series of responses, rather than summarized as a single categorical response. Additional components of gastropod movement including speed and variation between individuals can be assessed using fractal methods as described by Chapperon and Seuront (2010). Elucidating whether gastropod movement conforms to developed correlated random walk models (Byers 2001) may provide insight to whether observed responses are truly random or directed (Reynolds and Rhodes 2009).

The evolutionary significance of *Tarebia granifera* producing a chemical cue which deters naïve gastropods should also be considered. The responses of gastropods that have a shared evolutionary history with *T. granifera* may provide an interesting perspective. *Melanoides tuberculata* is closely related to *T. granifera*, however only the Asian morphs of this species share evolutionary history (Genner et al. 2004). Future work should aim to determine whether

South African populations of *M. tuberculata* contain alien morphs, which is highly suspected for the population at Amatikulu Aquarium Plants because of the international trades undertaken within their operations. Once this has been established, the variation in responses reported for *M. tuberculata* can be attributed to the varying degrees of shared evolutionary history. *T. granifera* is considered to have diverged from *Melanoides* around 4.9 Ma (Genner et al. 2007) and is placed within a *Melanoides* dominated subclade (Myers et al. 2000). Therefore the evolution of chemical cues in *T. granifera* but not in *M. tuberculata* from the same geographic origin should also be investigated.

Behavioural responses have not been previously reported for interactions among heterospecific competitors. Mechanistic models of spread which have been developed for *Tarebia granifera* (see Snider and Gilliam 2008 and Facon and David 2006) report rapid colonization rates between sites that are closer together. Incorporating the evidence and observations from this study would support these patterns, as chemical cues may be transported short distances and reduce biotic resistance by influencing the movement of native individuals. This study elucidates a mechanism of invasion that explains successful establishment as well as spread of introduced non-native species. The role of chemical cues in influencing movement between competitors may be more prominent than previously acknowledged, especially in the aquatic environment, and may explain a variety of spatial patterns if it is considered as a novel invasion mechanism.

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