

Research Paper

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Putting infection on the map: Using heatmaps to characterise within- and between-host distributions of trematode metacercariae

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Abstract

The location of parasites within individual hosts is often treated as a static trait, yet many parasite species can occur in multiple locations or organs within their hosts. Here, we apply distributional heat maps to study the within- and between-host infection patterns for four trematodes (*Alaria marcianae*, *Cephalogonimus americanus*, *Echinostoma* spp. and *Ribeiroia ondatrae*) within the amphibian hosts *Pseudacris regilla* and two species of *Taricha*. We developed heatmaps from 71 individual hosts from six locations in California, which illustrate stark differences among parasites both in their primary locations within amphibian hosts as well as their degree of location specificity. While metacercariae (i.e., cysts) of two parasites (*C. americanus* and *A. marcianae*) were relative generalists in habitat selection and often occurred throughout the host, two others (*R. ondatrae* and *Echinostoma* spp.) were highly localised to a specific organ or organ system. Comparing parasite distributions among these parasite taxa highlighted locations of overlap showing potential areas of interactions, such as the mandibular inner dermis region, chest and throat inner dermis and the tail reabsorption outer epidermis. Additionally, the within-host distribution of *R. ondatrae* differed between host species, with metacercariae aggregating in the anterior dermis areas of newts, compared with the posterior dermis area in frogs. The ability to measure fine-scale changes or alterations in parasite distributions has the potential to provide further insight about ecological questions concerning habitat preference, resource selection, host pathology and disease control.

Introduction

The factors that influence how parasites select their hosts are often complex and depend on the attributes of both the host and the parasite (Dick and Patterson 2007; Johnson et al. 2019; Sukhdeo and Sukhdeo 1994). Some parasites are generalists in their use of host individuals or species (Woolhouse et al. 2001), such as the apicomplexan *Toxoplasma gondii* that can infect nearly all warm-blooded vertebrate taxa, from wolves (*Canis lupus*) to Hawaiian monk seals (*Monachus schauinslandi*) (Dubey 2021; Dubey et al. 2020). However, many parasites exhibit specificity for hosts as a function of species identity or individual host characteristics (e.g., sex, life stage or body size) (Johnson et al. 2019; Johnson and Hoverman 2014; Lewis et al. 2002; Manzoli et al. 2021; Sukhdeo and Sukhdeo 1994) (see Combes (1991) for a review of the encounter and compatibility filters between parasites and their host). Furthermore, the relative degree of host specialisation offers opportunities as well as challenges. Although specialists may be highly adapted to thrive within their hosts, they are often vulnerable to environmental changes that alter the availability or abundance of such hosts (Colléony and Shwartz 2020; Kellermann et al. 2009).

Many parasites also show remarkable specificity in their distribution *within* individual hosts or the microhabitat. We define a parasite microhabitat as a small area or niche region of habitat within the surrounding or larger habitat (e.g., host; Griffin et al. 2017; Holmes 1973; Marcogliese 2005; Rohde 1994). Microhabitat preferences may be selected to maximise initial infection success, total growth and reproduction or subsequent transmission (Montgomery and Roloff 2017; Poulin 2005; Sukhdeo and Sukhdeo 1994). Previous studies of parasites have highlighted the importance of host microhabitat (i.e., within-host factors), such as the local availability of resources, intra- and interspecific competition, immune defenses, temperature and pH (Friesen et al. 2018; Holmes 1973; Jensen and Johnsen 1992; Zolovs et al. 2018). For example, the nematode *Heligmosomoides polygyrus* specifically establishes within regions of the mouse small intestine with the longest villi, thereby providing worms with greater attachment opportunities (Bansemir and Sukhdeo 1994). Similarly, *Ixodes* spp. ticks exhibit preferential attachment to the head of avian hosts where shorter feathers may facilitate longer feeding duration and limit host grooming (Fracasso et al. 2019). Understanding parasites' use of microhabitats allows for deeper understanding of species-level interactions ecologically and evolutionary interactions between hosts and parasite.

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Importantly, parasites' use of microhabitat locations within their hosts can also change in response to environmental factors (Fretwell and Lucas 1969; Graham 2008; MacArthur 1958; Pederesen and Fenton 2007; Poulin 2007), rather than being static. Factors such as host habitat use, host health and coinfection status can directly or indirectly influence the within-host distribution of parasites (Bashey 2015; Bell et al. 2006; Johnson et al. 2014; Mideo 2009). For instance, the same nematode referenced above (*H. polygyrus*) in mice has been reported to change its within-host distribution depending on the host's food source, such that if the host is fed a low-protein diet the parasites migrate anteriorly in the small intestine in search of more resources (Bawden 1969). Parasites' within-host distributions can also shift with age or condition of the host. For instance, the parasitic copepod *Lernaeocera branchialis* moves from anterior to posterior gill locations as their fish hosts age, likely offering better access to host's heart (Smith et al. 2007). Alterations in parasites' selection of microhabitats within the host can result from competition between parasites for nutrients, intestinal space and/or attachment sites (Poulin 2007). In one example, Holmes (1957, 1961) used experimental infections of rats to show that the distribution of acanthocephalan parasites shifted posteriorly in the intestine when hosts were coinfecting with a species of cestode. Furthermore, parasite-mediated competition can shape free-living community structure and host species distribution. For instance, Friesen et al. (2018) experimentally investigated changes in microhabitat selection and found when a host was infected with two different isopod species within the genus *Austroditoe*, the isopods altered their microhabitat compared to when infected with a singular parasitic infection. Therefore, understanding within-host distribution of microhabitats and load can be especially important for parasites that use multiple transmission routes.

Digenetic trematodes offer interesting insights into parasite habitat selection because their life cycles involve multiple hosts interacting at different spatiotemporal scales, for which each habitat (host species) or microhabitat (within-host infection location) can directly affect the parasites' survival from stage to stage (Combes 1991). Specifically, trematodes go through a series of juvenile/intermediate stages (i.e., miracidium, sporocyst, redia, cercaria, metacercaria/mesocercaria) before developing into an adult within the definitive host(s) (Schell 1985). Movement between host species is often through a combination of free-living infective stages followed by trophic transmission, in which the definitive host consumes infected intermediate host(s) and acquires the parasite stages of its meal (Combes et al. 2002; Esch and Fernandez 1994; Schell 1985). Different species of trematodes have been reported across nearly every host organ, tissue, muscle and blood vessels (LaRue 1951; Schell 1985). Of particular interest for habitat selection are trematodes with second intermediate hosts, for which their free-living cercariae often 'choose' a location or tissue to invade before establishing as a metacercaria/mesocercaria in less than 24 hours (Johnson et al. 2019; Stewart Merrill et al. 2022; Sukhdeo and Sukhdeo 1994; Szuroczi and Richardson 2009). This habitat choice is informed by physical and chemical cues associated with the host and the surrounding environment (Johnson et al. 2019; Leung et al. 2010; Sears et al. 2013). The consequences of microhabitat selection can influence host pathology as well as the likelihood that infected hosts are consumed by definitive hosts. For example, *Dicrocoelium dendriticum* is transmitted to ants when they ingest snail mucus containing cercariae; while most cercariae establish in the crop of their ant host, one cercaria travels to the gnathal ganglion instead where it induces the behavioral alterations

that increase the likelihood the ant is consumed by the parasite's sheep definitive host (Martín-Vega et al. 2018). Additionally, parasite density within specific microhabitats has potential consequences for the host, especially for cases where pathology is a function of infection intensity and location. For instance, aggregation of diplostomatid trematodes within the eyes of fish can disrupt vision and reduce host fitness (Griffin et al. 2017; Grobbelaar et al. 2015; Seppälä et al. 2005). Observations such as these highlight the importance of understanding fine-scale variation in parasites' use of microhabitats within their hosts.

Heatmaps are powerful visual tools that use a colour-grid system to display data across a variety of applications. Heatmaps were developed in 1873 by French economist Toussaint Loua to display and summarise changes in human population density over time for 20 districts in Paris. Later in 1914, 'clustering' techniques were used in heatmaps that presented rankings of the United States educational features of testing efficiency (Wilkinson and Friendly 2009). Contemporary heatmaps are graphical representations for pattern visualisation that summarise and communicate patterns through space and/or time. The colourful and intuitive nature of heatmaps makes them well-suited for presentations of high-throughput data because millions of rows and columns can be displayed on a single map (Gehlenborg and Wong 2012). Heatmaps are commonly used to illustrate weather patterns, population maps and financial trends. In the biological sciences, they are used in an array of applications like depicting gene expression, hierarchical cluster trees and surveillance and prevention of disease (Eisen et al. 1998; Gehlenborg and Wong 2012; Kaspi and Ziemann 2020). Thus far, however, there are fewer applications of heatmaps in the field of parasitology, where they could have extensive utility in tracking infections through time and visualising infection across a landscape. van Beest and colleagues (2019) recently utilised heatmaps to illustrate the common entry points of *Cardiophaloides longicollis* (Rudolphi 1819) cercariae in gilthead seabream (*Sparus aurata*), where they showed that cercariae select strategic entry portals to reach the fish brain. Other recent examples have used heatmaps to graph observed infection of parasites within fish gills, including comparisons between each gill arch and the structures of the gills (Oliveira et al. 2022; Thys et al. 2022). Twumasi et al. (2022) further used heatmaps and mathematical modeling to visualise how the microhabitats used by monogenean parasites (*Gyrodactylus* spp.) in fish hosts shifted over time and in response to host type. Regardless of data complexity, heatmaps facilitate data visualisation that quickly communicates patterns to the viewer using easy-to-understand colour gradations.

Here, we used heatmaps to illustrate the fine-scale distribution of trematode metacercariae within and between amphibian host taxa (*Pseudacris regilla* and *Taricha torosa* and *T. granulosa*; hereafter, *Taricha* spp.) and compared these patterns among four trematode species. Specifically, we documented the within-host locations of larval trematodes from 71 hosts collected from six sites within the Bay Area of California, USA. This included (1) distributional information for the trematodes *Alaria marcianae*, *Cephalogonimus americanus*, *Echinostoma* spp. and *Ribeiroia ondatrae* within the chorus frog host *P. regilla* and (2) comparative data for *R. ondatrae* between two amphibian taxa, *P. regilla* and *Taricha* spp. Specifically, we made an individual heatmap for each dissected amphibian-by-parasite combination and then used this information to generate visualisations for average infection loads. Thus, rather than treating infection locations as a fixed characteristic of the parasite, we applied a 1.7 × 1.7 mm grid network to each host and plotted the specific location of each larval trematode within the

host. We also compared the microhabitat distribution of *R. ondatrae*, a virulent parasite that is known to cause limb malformations, between a frog host (*P. regilla*) and newts (*Taricha* spp.), which rarely show parasite-induced malformations in nature (Johnson et al. 2012, 2013; Stewart Merrill et al. 2022). By detailing variation in parasite distributions within hosts and among species, heatmaps provide a tool for future work on a diverse range of topics, including pathogen transmission, intraspecific interactions, coinfection and pathology, particularly when involving multihost parasites important for wildlife conservation or human health (Gandon 2004; Gandon et al. 2002).

Materials and methods

Study system

The digenetic trematodes *A. marciana*, *C. americanus*, *Echinostoma* spp. and *R. ondatrae* can all use amphibians as second intermediate hosts (Calhoun et al. 2019; Johnson and Hoverman 2014; Sutherland 2005). Each of these parasites has a three-host life cycle with freshwater snails as the first intermediate host, amphibians as a possible second intermediate host and vertebrate definitive hosts (bird, mammal, amphibian or reptile), although there is variation among them. *Ribeiroia ondatrae* (Digenea: Echinostomatidae; Tkach et al. 2016) sequentially infects freshwater snails (family Planorbidae), amphibians and finally birds or, less commonly, mammals, as definitive hosts (Beaver 1939; Hannon et al. 2016; Johnson et al. 2004). Metacercariae of this parasite have been documented in 27 different amphibian species (Johnson and McKenzie 2009; Johnson et al. 2004, 2010; Keller et al. 2021). This parasite has gained notoriety for its capacity to alter the host and cause severe limb malformations (Blaustein and Johnson 2003; Goodman and Johnson 2011a, 2011b), delay host growth (Johnson et al. 2006; Romansic et al. 2011) and induce acute mortality of the larval amphibian host (Johnson and Lunde 2005; Keller et al. 2021; Wilber et al. 2020). Metamorphic amphibians with limb deformities caused by *R. ondatrae* encystment are hypothesized to be more prone to predation by definitive hosts, potentially enhancing trophic transmission (Goodman and Johnson 2011a).

Species of *Echinostoma* (Digenea: Echinostomidae; Tkach et al. 2016) have complex life cycles often involving freshwater snails, amphibians, mammalian and/or avian hosts (Johnson and McKenzie 2009). Free-swimming cercariae emerging from pulmonate snails (including species in Planorbidae, Physidae, Lymnaeidae) infect the developing kidneys of larval amphibians, wherein they form metacercariae. *Echinostoma* spp. metacercariae have been recorded from at least 29 different amphibian hosts (Calhoun et al. 2017; Johnson et al. 2014; Stewart Merrill et al. 2022). Interestingly, *Echinostoma* spp. cercariae can also form metacercariae in freshwater snails, which function as a second intermediate host (Beaver 1937).

Cephalogonimus americanus (Digenea: Cephalogonimidae) uses adult amphibians and reptiles as definitive host and amphibians as second intermediate hosts (Calhoun et al. 2017; Dronen and Lang 1974). Specifically, eggs from adult worms are released into the water where a snail becomes infected; inside the snail, sporocysts release xiphidiocercariae that penetrate larval amphibians and form metacercariae in the skin (Calhoun et al. 2017; Dronen and Underwood 1977; Schell 1985). Although planorbid snails of the genus *Helisoma* serve as the main molluscan host in North America, species of *Lymnaea* function as first intermediate hosts for species in Europe (Dronen and Underwood 1977). Seven species

of *Cephalogonimus* are known to occur in amphibians in North America (Ubelaker and Kimbrough 1970).

Alaria marciana (Digenea: Diplostomatidae) also uses a three-host life cycle but may further include an additional paratenic host(s) (reviewed in Möhl et al. 2009). Sporocysts of *A. marciana* develop in planorbid snails, such as *Helisoma* spp. and release furcocercariae (forked-tail cercariae) that penetrate a larval anuran to form an unencysted mesocercariae (Hannon et al. 2017; Schell 1985; Shoop and Corkum 1981). Almost any vertebrate that consumes an infected anuran can function as a paratenic (or transport) host (Möhl et al. 2009), in which mesocercariae accumulate but do not undergo further development until the paratenic host is consumed by a suitable definitive host, such as species of dog, cat, fox, lynx, weasels and otters (Bezerra-Santos et al. 2021; Diakou et al. 2021; Foster et al. 2009; Hiestand et al. 2014; Möhl et al. 2009).

Animal collection and dissection

To examine parasite distributions within and between hosts, we collected 51 metamorphic Pacific chorus frogs (*P. regilla*; Gosner (1960) range = 43–46) and 20 larval newts (*T. torosa* and *T. granulosa*; stage 4T–5T; (Wong and Liversage 2005)) from six ponds in the Bay Area of California and Lassen National Forest. Animals were collected during metamorphosis between June and August in 2020 and 2022. All ponds supported ramshorn snails (*Helisoma trivolvis*), which amphibians using a combination of dipnets and hand captures (Johnson et al. 2013) in accordance with University of Colorado IACUC permit 2613.

After collection, we humanely euthanised amphibians using an overdose of buffered MS-222 (dose = 1 g/500 ml of water), measured their snout–vent length (SVL) using digital calipers and examined all major organs and tissues for macroparasites with an Olympus SZX16 stereo dissecting microscope (Olympus Corporation, Tokyo, Japan). To record the specific location of each metacercaria or mesocercaria, individual tissues and organs were isolated into a gridded Petri dish. Parasite location and intensity were carefully recorded onto a standardised, two-dimensional anatomical diagram using a digital tablet (Samsung tablet model SM-P610). Specifically, images were captured either by the authors (for *Taricha*) or purchased for use by another photographer (for *Pseudacris*; copyright M. Benard). Each dissection was also conducted in a standardised sequence, beginning with the skin of the hind limbs, followed by tail resorption site and then the muscles in the hind legs. Next, we examined the skin in the anterior region of the frog including the head, followed by the mandible, tongue and muscles in the forelimbs and pectoral girdle. Finally, we opened the host to examine the internal organs and body cavity. Parasites were identified and counted under 60–200× magnification using the keys of Lehmann (1954), Schell (1985) and Gibson et al. (2002) on an Olympus BX51 compound scope (Olympus Corporation, Tokyo, Japan). Detailed amphibian necropsy methods are provided in Calhoun et al. (2019), Riepe et al. (2019) and Johnson et al. (2018). Parasite average load was calculated as the total number of parasites divided by the number of dissected hosts, while average intensity was calculated as the total number of parasites divided by the number of infected hosts for the specific taxa of interest (Bush et al. 1997).

Heatmap development

To generate a standardised, distributional heatmap illustrating the location of each individual parasite within the hosts, we applied a 1.7 × 1.7 mm grid network to the two-dimensional representation

of a frog or newt silhouette and recorded both the presence and number of metacercariae/mesocercariae in each cell. We selected this grid cell size to incorporate the sizes of all studied parasites including its surrounding cyst. In some cases, individual grids might contain multiple parasite individuals of the same or different species. After creating heatmaps for individual hosts, we further calculated average infection load for each parasite species by calculating the mean number of metacercariae/mesocercariae per grid cell from across all dissected hosts infected by a specific trematode. Using the program Affinity Photo (Serif Europe Ltd., Nottinghamshire, United Kingdom), we joined the frog image with the loads for individual hosts or averaged values and applied colours corresponding to varying degrees of load (approximately following colours used in the ArcGIS guide (Nelson, 2018)). The same amphibian image was used for every host of that species to ensure a standardised field of view with corresponding grid cells. To create a glowing effect for each point, we used the round soft brush (size 64) for outer colour and central points (size 32). To demonstrate increased load, the outer and central brush size increased as the load of parasite per grid cell increased. To allow for comparable heatmaps across parasite taxa and hosts, we shifted the central colour in points to reflect increases in the average infection of a cell. In general terms, load is reflected by variation in the size of the point, its colour and colour of the border, such that a cell that was more commonly infected is depicted by a large, yellow point with red outer ring. As the average load increases, the red outer ring diminishes, and the point appears more yellow on the heatmap. More specifically, we doubled the brush size across different bins for infection frequency (i.e., 0 to 9%, 10 to 19%, 20 to 29%, etc.). The same technique was used for all heatmap figures (see below).

Results

Overview of infection patterns among ponds and species

All 51 *P. regilla* were infected with metacercariae of one or more species of trematode. Some hosts were infected with a single trematode species ($n = 9$), two species ($n = 38$) or three species ($n = 4$); no animals were infected with all four parasites examined. Specifically, 71% of animals were infected with *R. ondatrae*, 61% with *C. americanus*, 55% with *A. marciana* and 50% with *Echinostoma* spp. Dissected frogs varied in load, range and average intensity when compared across sites (see Table 1 and Figure 1). For instance, *A. marciana* had the greatest average load (20.1 ± 6.2 SE), average intensity (33.1 ± 9.5 SE) and maximum load (214), whereas *C. americanus* had the lowest average load, average intensity and maximum load (1.9 ± 0.5 SE, 5.1 ± 1.1 SE, 17).

Of the 20 *Taricha* collected (10 of each species from a single pond), all were infected with *R. ondatrae* (Figure 2). The intensity of *R. ondatrae* from infected animals across both species was 34.6 ± 5.0 SE. *Taricha granulosa* supported lower average load (27.9 ± 7.2 SE)

when compared with *T. torosa* (41.3 ± 6.3 SE). Between hosts, *Taricha* spp. supported a larger average load (34.6 ± 5.0 SE) compared with *P. regilla* load of 17.7 ± 4.2 SE (although note that these species were not collected from the same pond). No other trematode species were detected within the sampled newts.

Within-host distribution of larval trematodes in amphibians

Among trematode species, the distribution of metacercariae across hosts and ponds varied both with respect to the primary location used by a parasite species and the relative degree of specificity/consistency (view Figure 1 [for regions of frogs] and Figure 3 [heatmap]). In *P. regilla*, mesocercariae of *A. marciana* were more common in the anterior (head) portion of the frog ($38.2\% \pm 2.1\%$ SE of average proportion of parasites per location) relative to the posterior portion ($8.3\% \pm 2.1\%$ SE), with little to no infection in the limbs (Figure 3A). In general, *A. marciana* was found in the subcutaneous tissues of specific organs or organ systems. Specific areas where mesocercariae were detected in *P. regilla* included (average proportion of parasites per location): mandible inner dermis ($19.0\% \pm 2.3\%$ SE), chest and throat inner dermis ($12.4\% \pm 2.0\%$ SE), posterior outer epidermis ($8.3\% \pm 2.4\%$ SE), anterior outer epidermis ($6.6\% \pm 1.9\%$ SE) and tail reabsorption area (TRS) inner dermis (less than 1% of average mesocercariae). Specifically, a prominent band of heavy infection occurred near the midline of the anterior skin, across the transition from head to body of the frog (Figure 3).

Infection by *C. americanus* occurred predominantly at the anterior and posterior extremes of the host body, especially around the mandibular region and the TRS (Figure 3B). Similar to *A. marciana*, metacercariae were not detected distally into the limbs of infected hosts but were found evenly distributed between the anterior and posterior regions of the body. Specific areas of infection included TRS inner dermis ($35.3\% \pm 8.3\%$ SE), chest and throat inner dermis ($24.4\% \pm 7.1\%$ SE), mandible inner dermis ($24.4\% \pm 7.1\%$ SE), posterior outer epidermis ($11.8\% \pm 5.3\%$ SE) and anterior outer epidermis ($3.9\% \pm 1.9\%$ SE).

In *P. regilla*, both *R. ondatrae* and *Echinostoma* spp. infection distributions were concentrated within specialised regions (see Figures 3C and 3D). *Ribeiroia ondatrae* infections occurred most commonly in the posterior region of the frog ($92.0\% \pm 1.8\%$ SE), with a secondary cluster of infections congregating near the distal edge of mandible ($5.0\% \pm 1.4\%$ SE). Specifically, *R. ondatrae* was detected in the following locations: posterior outer epidermis ($55.5\% \pm 5.0\%$ SE), TRS inner dermis ($36.3\% \pm 4.6\%$ SE), mandible inner dermis ($5.0\% \pm 1.4\%$ SE), anterior outer epidermis ($1.1\% \pm 0.4\%$ SE) and chest and throat inner dermis ($1.6\% \pm 0.8\%$ SE). *Echinostoma* spp. metacercariae were localised within the host kidneys ($93.3\% \pm 1.6\%$ SE), particularly in the right kidney ($61.3\% \pm 3.0\%$ SE) compared to left ($32.1\% \pm 3.7\%$ SE). Within that microhabitat, *Echinostoma* spp. tended to congregate in the

Table 1. The overall average load, intensity (± 1 SE), and infection range of four trematode taxa detected in *P. regilla* collected within freshwater ponds California from 2020–2022.

Trematode	Number of infected hosts	Average load $\pm$ SE	Average intensity $\pm$ SE	Infection range
<i>Alaria marciana</i>	31	20.10 ± 6.22	33.06 ± 9.53	0–214
<i>Cephalogonimus americanus</i>	19	1.92 ± 0.54	5.16 ± 1.10	0–17
<i>Echinostoma</i> spp.	25	17.04 ± 5.20	31.04 ± 8.61	0–220
<i>Ribeiroia ondatrae</i>	25	17.74 ± 4.18	24.84 ± 4.17	0–77

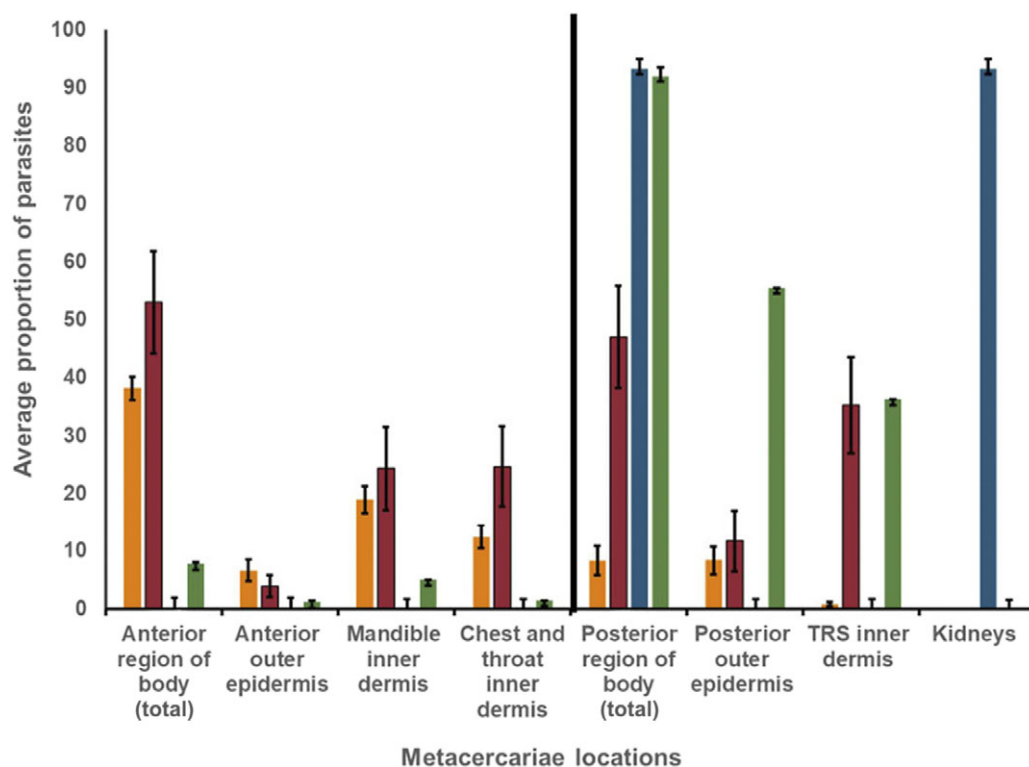


Figure 1. Average proportion of parasites (± 1 SE) of four trematodes (*A. marciana*, *C. americanus*, *Echinostoma* spp. and *R. ondatrae*) by location within the host for 51 *P. regilla* in California. Location categories are as follows: posterior outer epidermis, anterior outer epidermis, tail reabsorption area (TRS) inner dermis, mandible inner dermis, chest and throat inner dermis and kidneys. Categories to the left of the black vertical line represent anterior locations, while those to the right of the line are from posterior locations.

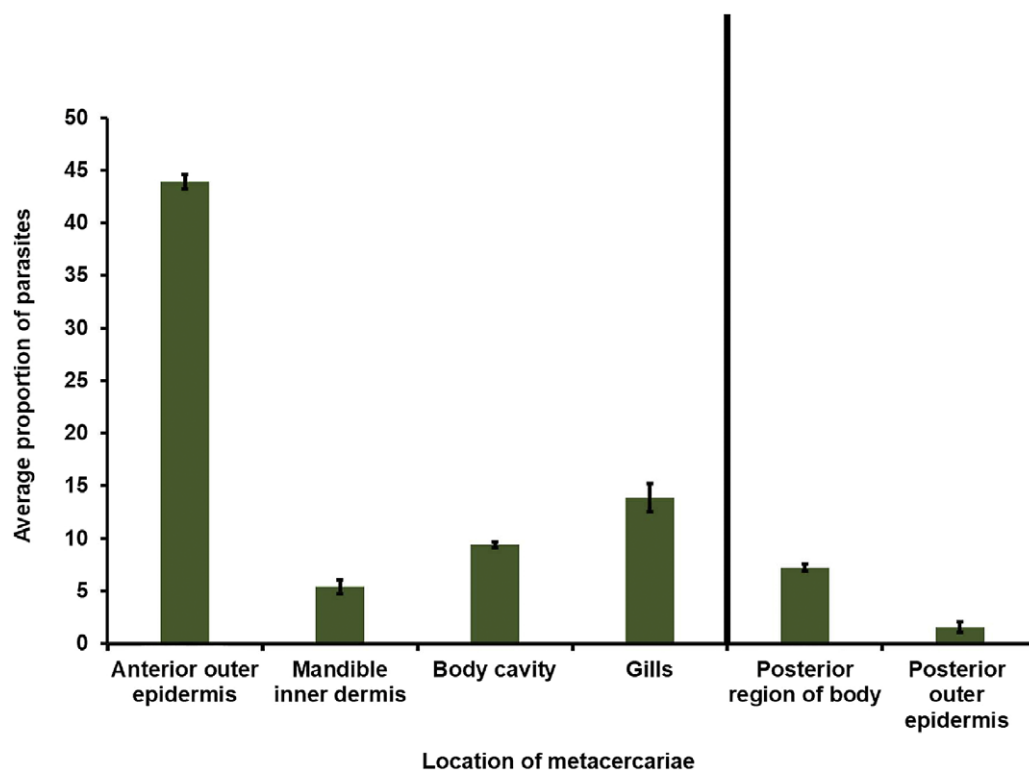


Figure 2. Average proportion of parasites (± 1 SE) of *R. ondatrae* within *Taricha* spp. collected in a freshwater pond in California in 2022. Location categories are as follows: posterior outer epidermis, anterior outer epidermis, mandible inner dermis, body cavity and gills. Categories to the left of the black vertical line represent anterior locations, while those to the right of the line are from posterior locations.

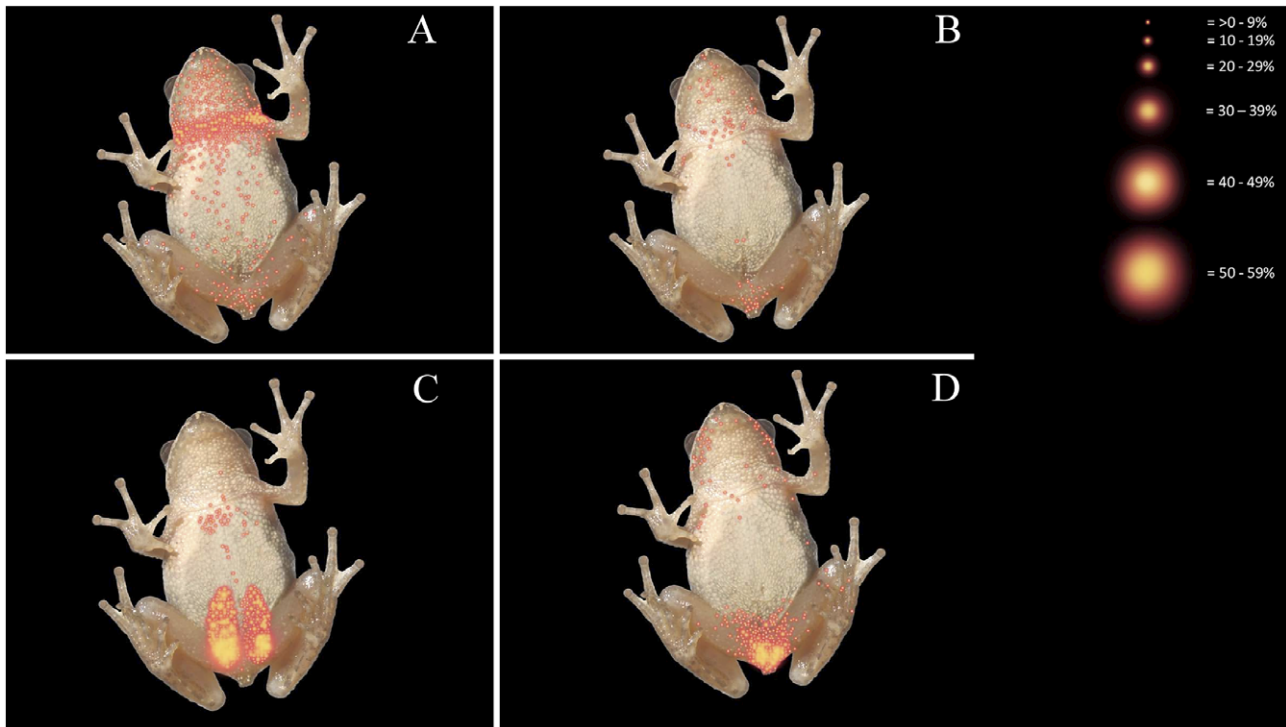


Figure 3. *Pseudacris regilla* trematode distributional heatmaps where each dot represents the average infection prevalence per grid cell for an individual trematode species. A maximum value of 100% would indicate that a specific grid cell was always infected with one or more metacercariae across all examined hosts. Individual trematode taxa are as follows: A) *A. marciana*, B) *C. americanus*, C) *Echinostoma* spp. and D) *R. ondatrae*. Low infection prevalence (less than 10% average infection) is represented by small dots with red outlines and small yellow centers whereas dots with larger yellow centers and small red outlines represent increased infection prevalence. For specific infection ranges see key. Chorus frog image by M. Benard (used with permission).

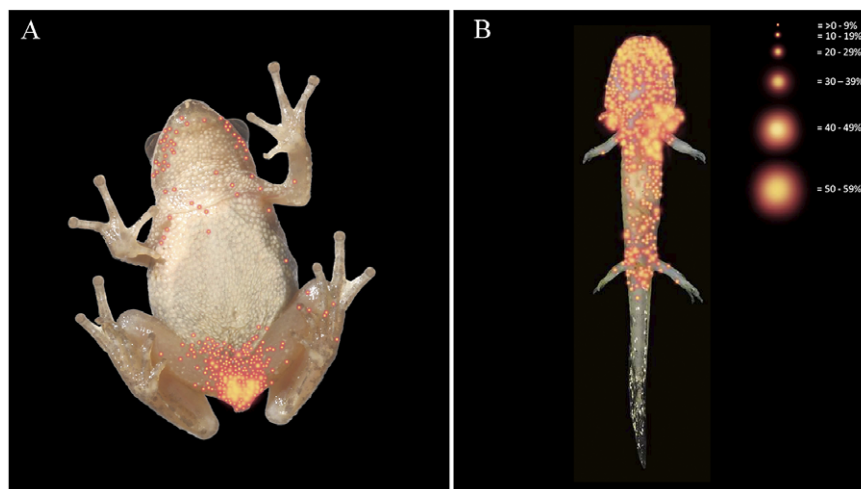


Figure 4. *Pseudacris regilla* (A) and *Taricha* spp. (B) *R. ondatrae* distributional heatmaps where a heatmap dot represents the average infection prevalence per grid cell for *R. ondatrae*. Light infection prevalence (less than 10% average infection) is represented by small dots with red outlines and small yellow centers whereas dots with larger yellow centers and small red outlines represent increased infection prevalence. For specific infection ranges see key.

posterior half of the kidney and towards the center, with lighter infection on the margins of the kidneys.

Finally, in newts (*T. torosa* and *T. granulosa*), *R. ondatrae* metacercariae loads were higher in anterior regions of the body ($86.1\% \pm 1.3\%$ SE; Figure 3B), for which the highest average load occurred in the mandible inner dermis ($43.9\% \pm 0.7\%$ SE; Figure 2). Comparing the total detected metacercariae found in newts, a total of 32.8% were found in the anterior outer epidermis and $9.4\% \pm 0.3\%$ SE in the gills. Fewer than 10% of total detected metacercariae were found in the tail outer epidermis and posterior outer epidermis.

Between-host infection distribution

Examination of *R. ondatrae* within *P. regilla* and *Taricha* indicated differences in average load, intensity of infection and the primary locations within the host where infections were concentrated (Figure 4). Although the majority of metacercariae in *P. regilla* were in the posterior region of the host (92.0%), especially around the tail reabsorption site, *Taricha* spp. infections were concentrated towards anterior host tissues (86.1%) with very few metacercariae in the posterior region (13.8%). *Taricha* spp. infections were

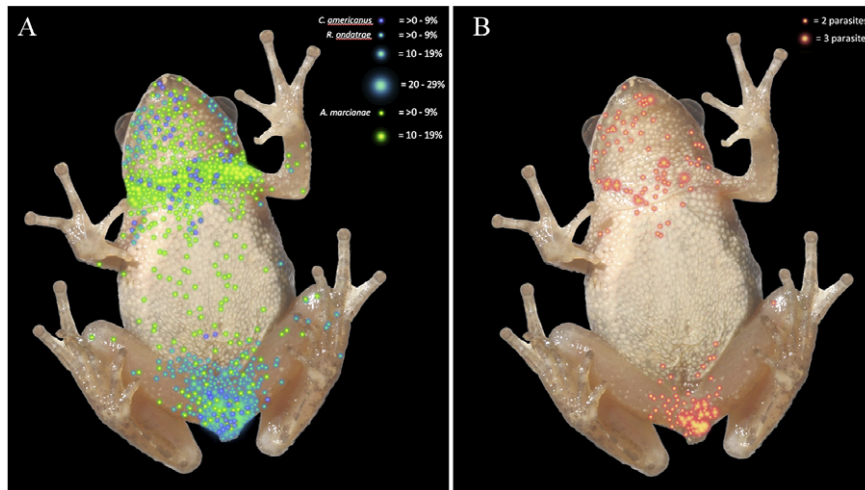


Figure 5. A) *Pseudacris regilla* outer and inner dermal trematode distributional heatmaps where a heatmap dot represents the average infection prevalence per grid cell for individual trematode species. Individual trematode distribution are as follows: *A. marciana* lime green, *C. americanus* purple and *R. ondatrae* as teal. For specific ranges of infection prevalence for each trematode see key in Figure 5A. Similar to Figures 3 and 4, low infection prevalence (less than 10% average infection) is represented by small dots for each colour, while larger dots for each colour represent increased infection prevalence. B) Areas of overlap infection between two or three trematode species within the outer or inner dermis, where larger heatmap dots represents areas with overlapping infection of all three trematodes genera were detected within a grid cell, and smaller dots represent a combination of overlap infection of any two trematodes within a grid cell.

commonly found in anterior outer epidermis, gills and mandible inner dermis (Figure 4B). Similarly, even while infections could be common at the base of limb structures and the base of the tail or TRS inner dermis, neither genera of amphibians supported *R. ondatrae* infections in the distal limb tissue. Although the load of metacercariae was heavier in *Taricha* spp. compared with *P. regilla*, *P. regilla* supported a higher maximum load per grid cell (50–59%) compared with *Taricha* spp. (20–30%). Meaning that *R. ondatrae* metacercariae in *P. regilla* aggregated in the same location more commonly than in *Taricha* spp.

Overlapping distribution

The generated heatmaps of hosts infected with trematodes also revealed areas of spatial overlap among parasite taxa within coinfecting hosts, such that two or more parasites often occupied the same or adjacent grid cells. These overlapping areas are of interest as they have the potential to reflect interactions between co-occurring parasites. Specifically, overlapping distributions of infection occurred between *A. marciana*, *C. americanus* and *R. ondatrae*, either as a combination of two- or three-way overlaps (Figures 5A and 5B). In general, the distribution of *A. marciana* in *P. regilla* overlapped around the chest and mandible inner dermis with *C. americanus* and around the mandible inner dermis with *R. ondatrae*. Figure 5B provides a clear view of parasite overlap, showing that two parasite overlaps most commonly occur in the mandible inner dermis, TRS inner dermis and chest and throat inner dermis. However, three-way overlaps were less common when compared to two-way overlaps in *P. regilla* (Figure 5B).

Discussion

Our study highlights the utility of heatmaps as an illustrative tool for examination and understanding of parasite habitat use. Historically, parasite location or distribution within a host is often treated as a static, categorical value, which can limit opportunities to explore fine-scale variation of the within-host distributions of parasites.

Here, we provide heatmaps that display parasite habitat and microhabitat use within *P. regilla* and between *Pseudacris* and *Taricha* to examine parasite distribution of common trematodes that vary in habitat specialisation. Within *P. regilla* hosts, trematodes varied in microhabitat use. Both *C. americanus* and *A. marciana* were relative generalists that often occurred throughout the host, whereas *R. ondatrae* and *Echinostoma* spp. were highly localised to a specific organ or organ system within the host. Heatmaps for *P. regilla* also indicated areas of high parasite overlap among trematodes, such as the epidermal and dermal parasites (*A. marciana*, *C. americanus* and *R. ondatrae*), suggesting that direct and indirect interactions could occur. Parasites' use of host tissues and locations also differed between host taxa. For *R. ondatrae*, for instance, metacercariae in newts (*Taricha* spp.) were primarily detected in the anterior dermal tissues and organs, whereas infections in chorus frogs (*Pseudacris*) were concentrated in posterior dermal tissues. With a quantitative estimate of relative frequency by locations, we can begin to uncover the underlying mechanisms in coinfection, habitat resource partition and parasite-induced pathology.

The highly pathogenic trematode, *R. ondatrae*, exhibited a specialised distribution in which metacercariae were concentrated in the posterior dermal regions of the frog specifically around/or near the limb development regions. This finding is consistent in wild caught and experimental animals within the literature (Johnson and Hoverman 2012; Johnson et al. 2004; LaFonte and Johnson 2013), including within hosts that have been experimentally anaesthetised prior to exposure (Hannon et al. 2017; Paull et al. 2012). Given the short time span afforded to *R. ondatrae* cercariae to find a suitable host and encyst (<24 h), microhabitat specialisation is all the more intriguing. Interestingly, the microhabitat preference of *R. ondatrae* to the hind limbs in anurans has even been documented in experimental anaesthetised tadpoles (Daly and Johnson 2011) as well. The implications of *R. ondatrae* microhabitat distribution can be directly linked to host pathology because *R. ondatrae* exposure causes limb malformations in many amphibian species in a dose-dependent manner (Johnson et al. 2004, 2011, 2012).

Parasite microhabitat distribution differed in hosts such that *R. ondatrae* specialised in the posterior dermal regions of the frog

while, in larval *Taricha R. ondatrae* inhabited the chest, throat and the mandible inner dermal regions of the animal. These locations are echoed in the literature in similar taxa of animals like salamanders (Johnson et al. 2006, 2012; Keller et al. 2021). Furthermore, this discrepancy in microhabitat could similarly explain discrepancies in malformation levels between anurans and caudates. Exposure of caudate larvae to *R. ondatrae* cercariae, even at relatively high dosages, is less likely to induce malformations, which is consistent with the parasite's less limb-specific distribution within these hosts. Without metacercariae development in the hind limbs it's unlikely that the parasites unique pathological changes in the host will occur.

Similarly, the metacercariae of *Echinostoma* spp. were also concentrated in specific microhabitat location, in this case specifically infecting the kidneys. This is well supported in the literature as the primary microhabitat location of *Echinostoma* spp. metacercariae (Holland et al. 2007; Johnson et al. 2014; Orlofske et al. 2009). *Echinostoma* spp. cercariae travel up the cloaca, migrate through the mesonephric ducts to the kidney tubules and finally encyst in the kidney (Fried et al. 1997; Johnson and McKenzie 2009; Martin and Conn 1990). Like *R. ondatrae*, *Echinostoma* spp. demonstrates a clear cercariae choice of an infection location. Interestingly, both *R. ondatrae* and *Echinostoma* spp. have large cercariae, commonly infect the posterior regions of the frog, and cause pathology (Johnson and McKenzie 2009). Whether this infection pattern leads to malformation or is reducing hydrodynamic drag on the infected frogs (Daly and Johnson 2011; Goodman and Johnson 2011b; Taylor et al. 2005) is unknown. In our study the highest load of *Echinostoma* spp. metacercariae occurred in the posterior right kidney, which is similar to the previous studies (Holland 2009; Orlofske et al. 2009; Thiemann and Wassersug 2000a). Johnson and colleagues (2014) found that in over 6,000 hosts across nearly 30 species of frogs, 62% of echinostomes were found in the right kidney. Interestingly, they did not see the follow-the-leader hypothesis because their results did not show an increase in bias with infection intensity. This pattern was driven by positional differences between the two kidneys because the right kidney sits more posterior within the body due to displacement by the liver.

Cephalogonimus americanus and *A. marciana* were both found to be more generalists in the distribution of their metacercariae/mesocercariae within amphibian hosts. One explanation for general distribution is that *Alaria* spp. and *Cephalogonimus* spp. cercariae are both small in body size compared to *Ribeiroia* or *Echinostoma*, perhaps invoking less host response during tissue penetration and less chance of getting scratched off by anti-parasite behaviors so therefore can infect more places within the host (see Sears et al. (2013)). We found metacercariae of *C. americanus* were often localised on the poles of the frog (e.g., mandible inner dermis and TRS inner dermis), while *A. marciana* mesocercariae were most commonly recorded around the anterior head region, chest and throat inner dermis, body cavity and TRS inner dermis. To some extent, the concentrations that were observed for parasites such as *C. americanus* may be influenced by morphological and hydrodynamic properties of the host (Daly and Johnson 2011; Goodman and Johnson 2011b; Taylor et al. 2005). For instance, if the cercariae are nonspecific about penetration location, a substantial number may end up in the TRS because this represents the tissue formerly found in the tadpole tail. As the tail reabsorbs, metacercariae presumably 'ride up' and become concentrated in the TRS (see Thiemann and Wassersug (2000b)). Metacercariae can also become concentrated around the mandible and head as cercariae from the water column are 'inhaled' by the tadpole; they

may also be less likely to be pushed away here relative to locations closer to the siphon and the tail. Observational evidence suggests that *A. marciana* cercariae are nonspecific about where they penetrate the tadpole host and others have detected *Alaria* spp. in various taxa of frogs and report a wide range of distribution (Buller 2013; Voelkel et al. 2019). Interestingly, the distribution of *Alaria* spp. within amphibians may vary with infection load; for instance, Voelkel (2019) reported that mesocercariae were aggregated around the eyes of their hosts but only among frogs with high infection loads (>100 mesocercariae per frog). However, *Alaria* species form mesocercariae (unlike *Cephalogonimus* spp.), and they can continue to move after invading the host, which could lead to postinfection changes in microhabitat use (see Shoop and Corkum (1984)). Thus, aggregation around the chest and throat inner dermis could provide resource or metabolic advantages to mesocercariae, helping to explain the seemingly nonrandom distribution. Additional research here is needed, including the potential use of fluorescent labeling to investigate postinfection changes in microhabitat use (LaFonte et al. 2015; Leung et al. 2010).

One caveat of this study is that we are visualising parasites distributions at a particular time in space which may not be the result of the parasites choice but rather post settlement mortality. For this study, we examined parasites using classic necropsy techniques of larval amphibians across Gosners and *Taricha* stages (in 43–46 *P. regilla* and 4–5T *T. torosa*) in hopes to quantify and examine metacercarial distribution in pre-metamorphic animals. Additionally, within coinfecting hosts (as most of ours were), competition between parasite species can alter their use of space, in some cases forcing a species out of their optimal location into one that is less preferred (see Poulin (2001)). Our results could also be improved with the use of fluorescent dye marking, which has been used to track metacercariae over several months allowing for a clearer picture of final microhabitat choice (LaFonte et al. 2015; Leung et al. 2010). Understanding whether the distribution captured within our presented heatmaps are the final choice of the metacercaria is key when examining microhabitat choice, especially when examining distributions resulting in host pathology.

Future applications of heatmaps can help to answer additional questions surrounding parasites' use of host microhabitats. Explicit comparisons of parasite within-host distributions between hosts infected by a single parasite taxon versus when coinfecting could offer insights into whether interspecific competition affects infection location. This was not possible in the current study because hosts were wild-caught and typically coinfecting. Additionally, heatmaps could explore how distribution changes with infection load, including whether high loads lead to increased use of 'suboptimal' spillover locations. Shifts in location have important implications for host pathology. For instance, Johnson et al. (2023) recently used heatmaps to demonstrate *R. ondatrae* distribution changes detected in *P. regilla* from high elevation and low elevation sites. Interestingly, this distribution change was found to be linked to high virulence and increased malformations as well (Johnson et al., in press). Finally, using heatmaps to identify areas of shared resources, identified by high areas of coinfection within a host can assist in exploring complex questions.

Lastly, the use of a spatial analysis tool in combination with our grid cell heatmaps would provide a quantitative metric to tease apart the mechanism(s) underlining parasite distributions. Because heatmaps are an applied method for visualising spatial patterns applying a statistical component allows for quantitative analyses. Human health arenas have begun utilising such combinations (Nawaz and Curtis 2019; Nearchou et al. 2021). For example,

Nearchou and colleagues (2021) developed a heatmap spatial analysis that investigated heterogeneity of intratumors in lymphocytes and tumor buds and patients' heterogeneity. Together, these data were applied to prognostic risk models that aided in recovery plans for the patients or potential discovery of targets for treatment. For parasite distributions applying a spatial analysis tool to the heatmap may reveal answers to how the load of parasites within a host effects distribution.

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