Risk of predation as a promoting factor of species divergence in threespine sticklebacks (*Gasterosteus aculeatus* L.)

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Icelandic freshwater systems are geologically young and contain only six species of freshwater fish. As these species colonized Icelandic fresh waters they were presented with a diversity of unique, uncontested habitats and food resources, promoting the evolution of new behaviour strategies crucial to the formation of new morphs and speciation. To determine the likelihood that predation threat could affect the antipredator behaviour and possibly the sympatric divergence of prey populations, we analysed antipredator behaviour of seven groups of Icelandic threespine sticklebacks (*Gasterosteus aculeatus*): two marine groups, one group from a lake without piscine predators, and two polymorphic lake populations, each with two groups occupying unique habitats. Shoaling cohesion, school formation and duration, and vigilance in predator inspection/avoidance behaviour varied greatly among groups. The differences appeared to be related to the risk of predation as well as to opportunities and constraints set by the different habitats. Antipredator behaviour was especially pronounced and differed extensively in two polymorphic forms from the lake Thingvallavatn, where predation risk is very high. By keeping the two morphs separate in their respective habitats, high predation risk may be a contributing factor in promoting the habitat-specific divergence of *G. aculeatus* seen in the lake. This suggests that in situations where refuge habitats are spatially separated, the risk of predation may contribute to the evolution of separate sympatric forms of small fish such as *G. aculeatus*. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 82, 189–203.


INTRODUCTION

The reduction of gene flow and isolation between subpopulations as they specialize for a novel resource such as an alternative habitat (Maynard Smith, 1966; Rosenzweig, 1987), food source (Skúlason et al., 1993; Smith, 1993), microclimate (Koopman, 1949), or host species (Bush, 1975) will lead to speciation without the need for geographic isolation (Dobhansky, 1940; Bush, 1994). Many models of sympatric speciation are based on the idea that speciation can occur when individuals of a population have ready access to alternative habitats within a region (Maynard Smith, 1966; Johnson & Gullberg, 1998). These models generally assume that each individual in the population selects one alternative habitat and then lives and utilizes resources in that habitat almost exclusively (Johnson & Gullberg, 1998). This can create a biological barrier to interbreeding (Futuyma, 1986) and result in selection against intermediate phenotypes. Selection can be disruptive, acting uniformly on every individual, or divergent in two or more habitats, allowing the extreme phenotypes to exploit the habitats where they best fit (Kondrashov, Yampolsky & Shabalina, 1998).

Apart from alternative habitats, other physical and biotic factors can affect the likelihood of sympatric morph divergence and speciation, such as stability of alternative habitats, productivity, distribution of potential breeding sites and predation risk (Skúlason, Snorrason & Jónsson, 1999; Snorrason & Skúlason,
Whether the risk of predation is a selective force that accelerates or retards the process of speciation is still a matter of controversy (McPhail, 1969; Holt, 1977; Schluter, 1998). Some researchers have suggested that the absence of competitors and predators in novel environments may lead to high population densities of colonizers, and that this ‘ecological persistence’ allows them to avoid extinction long enough to evolve reproductive isolation (Mayr, 1963; Schluter, 1998). Others have argued that predation relaxes competitive pressures by reducing the density of competing prey species, allowing higher diversity of prey species than in predator-free environments (Holt, 1977). Holt (1977) modelled different levels of competition and predation in prey communities and concluded that in most natural communities patterns of abundance probably reflect both direct competition and ‘apparent competition’, under which prey compete for predator-free space. However, in natural communities in which direct competition is weak or entirely absent, apparent competition may play a primary role in the structure of the community. Other researchers agree that apparent competition may be a strong diversifying force (Brown & Vincent, 1992; Abrams, 2000). Doebeli & Dieckmann (2000) showed through modelling that apparent competition could lead to sympatric speciation through evolutionary branching as prey species developed specialized defence morphologies and behaviour. Predation has already been shown to be a strong selective force for male breeding colouration (McPhail, 1969; Endler, 1995), antipredator morphology (Walker, 1997), and feeding (Peeke & Morgan, 2000), schooling (Seghers, 1974; Magurran, 1999), and inspection behaviour (Magurran & Seghers, 1994) in several fish species.

Due to geographical isolation and the limited age of the freshwater systems (maximum age = 11 000 years), Iceland harbours only six species of freshwater fish, which often experience high intraspecific but low interspecific competition. Lakes in the neovolcanic zone often offer distinct benthic habitats, such as lava formations with deep rifts, benthic spaces that smaller fish can exploit, and areas in the structure of the community. Other researchers agree that apparent competition may be a strong diversifying force (Brown & Vincent, 1992; Abrams, 2000). Doebeli & Dieckmann (2000) showed through modelling that apparent competition could lead to sympatric speciation through evolutionary branching as prey species developed specialized defence morphologies and behaviour. Predation has already been shown to be a strong selective force for male breeding colouration (McPhail, 1969; Endler, 1995), antipredator morphology (Walker, 1997), and feeding (Peeke & Morgan, 2000), schooling (Seghers, 1974; Magurran, 1999), and inspection behaviour (Magurran & Seghers, 1994) in several fish species.

We examined shoaling density and antipredator behaviour of G. aculeatus from seven different sites in south-western Iceland to determine if sympatric and allopatric groups employed different antipredator tactics. Two volcanic lakes, Thingvallavatn and Frostastaðavatn (the suffix ‘vatn’ means lake in Icelandic), containing separate lava littoral and soft-bottom, vegetated habitats, were selected for studying sympatric divergence. G. aculeatus from these two habitats in Thingvallavatn have been found to differ in morphology and foraging behaviour, but less clear morphological and behavioural differences were observed in Frostastaðavatn (Doucette, 2001; Kristjánsson, 2001; Kristjánsson et al., 2002a). Based on diet analyses of Salvelinus alpinus, predation pressure on G. aculeatus is considerable in Thingvallavatn (Malmquist et al., 1992), but is less intense in Frostastaðavatn (B. Jónsson, pers. comm.). By comparing the antipredator behaviour of the sympatric groups within these two lakes, with that of two ancestral marine groups, and a group from a third lake containing no predators, we searched for selection signatures of predation risk and assessed its role in the process of morph formation and sympatric speciation in G. aculeatus.

MATERIAL AND METHODS

G. ACULEATUS COLLECTION AND MAINTENANCE

During the summer of 1999, Gasterosteus aculeatus were collected from seven sites throughout Iceland (Table 1). Thingvallavatn is a very large (83 km³), deep (mean depth = 34 m) lake in the south of Iceland (64°10′N, 21°10′W) (Adalsteinsson, Jónasson & Rist, 1992). Frostastaðavatn is a smaller (2.55 km²), shallow (mean depth = 6.5 m) lake located in the highlands of southern Iceland (64°03′N, 19°00′W). G. aculeatus were collected from both the littoral lava habitat and the soft-bottom habitat of each of these lakes. Specimens were also collected from a third lake, Sauraðvatn, a small (0.84 km²), very shallow (mean depth = 0.2 m) lake located in the Mýrar district in south-west Iceland (64°40′N, 22°10′W). Sauraðvatn has a uniform, vegetated benthic habitat, and contains only a monomorphic G. aculeatus population with no piscine predators. Two marine sites were included in the experimental design, and it was assumed that they resembled the original ancestral population of G. aculeatus prior to their immigration to freshwater systems. Stokksýr is on the south shore of Iceland near the estuary of the River Ölfusá (63°50′N, 21°04′W), which drains Thingvallavatn via the River Sog. Hvassahraun is on the south-western coast of Iceland (64°01′N, 22°08′W). G. aculeatus were usually collected using wire minnow traps (No. 12562, Canada Fishing and Tackle Sports Ltd; mesh size = 3.2 mm). The traps were
Table 1. Description of the seven sites where Gasterosteus aculeatus were collected

<table>
<thead>
<tr>
<th>Site</th>
<th>Predation risk</th>
<th>Depth (m)</th>
<th>Vegetation</th>
<th>Special Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thingvallavatn lava</td>
<td>High – piscivorous</td>
<td>1</td>
<td>Patchy green algae (e.g. Tetraspora sp.)</td>
<td>Lava rubble with subbenthic spaces; cold, stable spring inflow</td>
</tr>
<tr>
<td>(TLAVA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thingvallavatn</td>
<td>Very high – piscivorous</td>
<td>17</td>
<td>Nitella opaca (dense stands to height of 1 m)</td>
<td>No lava; N. opaca stands contain dense G. aculeatus populations surrounded by open water</td>
</tr>
<tr>
<td>soft-bottom</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(TSOFT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frostastaðlavatn</td>
<td>Moderate – recently introduced Salv. alpinus &amp; Salmo trutta</td>
<td>5</td>
<td>Minimal</td>
<td>Steep lava shoreline; very dense G. aculeatus population</td>
</tr>
<tr>
<td>lava (FLAVA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frostastaðlavatn</td>
<td>Moderate – recently introduced Salv. alpinus &amp; Salmo trutta</td>
<td>1–1.5</td>
<td>Nitella sp. common, Chara sp. &amp; Myriophyllum sp. patchy</td>
<td>No lava; shallow, sandy shoreline; very dense G. aculeatus population</td>
</tr>
<tr>
<td>soft-bottom (FSOFT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sauravatn (SAUR)</td>
<td>Minimal – no piscine predators</td>
<td>&lt;0.5</td>
<td>Dense; multiple species</td>
<td>Very shallow, marshy lake; G. aculeatus collected in vegetation at shoreline</td>
</tr>
<tr>
<td>(Control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stokkseyri (STOKK)</td>
<td>Unknown – presumed minimal</td>
<td>&lt;1</td>
<td>Fucus sp. &amp; dense blue-green algae</td>
<td>Isolated marine tide pools on lava shoreline</td>
</tr>
<tr>
<td>(Marine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hvassahraun (HVASS)</td>
<td>Unknown – presumed moderate</td>
<td>2</td>
<td>Sparse Fucus sp. &amp; other marine seaweeds</td>
<td>Marine inlet on lava shoreline</td>
</tr>
<tr>
<td>(Marine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

weighted, attached by line to surface buoys, and left in the water for approximately 24 h. Attempts to catch G. aculeatus with traps at the Thingvallavatn lava site were unsuccessful and they were collected at this site with the use of electro-fishing gear.

G. aculeatus were maintained in the field in 100-L containers aerated with battery-operated air pumps for up to 5 days and they were fed dried Tubifex worms daily. The containers were kept outdoors in a shaded area. Outdoor temperatures ranged from 10 to 15°C. The fish were taken to the freshwater aquarium of Hólar College, Iceland and immediately transferred to 80-L glass aquaria (L × W × H = 50 × 40 × 40 cm; flow-through water 2 L/min; temperature 10°C ± 1°C; photoperiod 16 : 8 h light : dark). G. aculeatus from each site were maintained separately and 45 adults were placed in each tank for approximately 4 weeks prior to the start of the experiments. They were fed daily dried Tubifex, frozen or dried chironomid larvae, or flake food. All G. aculeatus were kept under exactly the same conditions; any effects of tank enclosure would be the same for all populations.

In the predation experiments, two brown trout Salmo trutta (approximately 25 cm standard length) from a small local stream (65°44’N, 19°05’W) and one Salv. alpinus (approximately 25 cm standard length) from the local lake Vatnshlidarvatn (65°31’N, 19°37’W) were used as piscine predators. These three fish had been maintained for approximately 1 year in large 800-L tanks with other members of their respective populations in the freshwater aquarium at Hólar. The predators were fed aquaculture feed daily and had no experience of G. aculeatus while in captivity.

**SHOALING DENSITY METHODOLOGY**

Experimental trials were conducted in a large flow-through aquarium (L × W × H = 160 × 70 × 80 cm; water temperature 10°C ± 1°C; Fig. 1). The back and sides of the tank were covered with white plastic to minimize external disturbance and to make G. aculeatus more visible. A Plexiglas divider was placed 40 cm from one end of the aquarium (Fig. 1). Three rows of holes (3 mm in diameter) along the edges of the Plexiglas divider allowed the free flow of water through the divider and the passage of odours from the predators and G. aculeatus. A large mirror (L × W = 160 × 90 cm) was angled above the tank to permit three-dimensional observations. Markings on the front of the glass aquarium and strings across its top divided the aquarium into 96 cubes (L × W × H = 20 × 20 × 17.5 cm or 7000 cm³). The size of the grid was selected so as to represent approximately four G. aculeatus body lengths (4 × 5 cm). It is within this proximity of conspecifics that fish can be considered to be in a shoal (Pitcher & Parrish, 1993).
Lighting consisted of three 60-W incandescent lamps illuminating the tank from above and a 125-W halogen spotlight illuminating the tank from the front. No cover was provided in the tank for *G. aculeatus* because we wanted to determine how they would react to predators in an open-water situation, where they would be unable to seek refuge.

In the wild, fish discriminate between potential shoal mates on the basis of features such as body size (Ranta, Juvonen & Peuhkuri, 1992a; Ranta, Lindstrom & Peuhkuri, 1992b; Ward & Krause, 2001) and parasite load (Barber & Huntingford, 1995; Barber, Huntingford & Crompton, 1995). Only healthy, non-parasitized *G. aculeatus* were selected for these experiments. Two replicate groups of 22 *G. aculeatus* from each site were tested. Given that fish typically form shoals based on body size, the first replicate group consisted of the largest *G. aculeatus* from the population sample and the second group consisted of *G. aculeatus* of a slightly smaller size (Table 2). They were tested in groups by site. Gender is not typically considered in studies of shoaling (e.g. Pitcher & Parrish, 1993; McRobert & Bradner, 1998; Ward & Krause, 2001), and gender differences were not considered in this study. Our experiments were conducted outside of the breeding season for *G. aculeatus* in Iceland, so any

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**Figure 1.** Experimental set-up for shoaling experiments. In the first set of trials, no predatory fish were present to the left of the transparent divider. In the second set of trials, two brown trout (*Salmo trutta*) and one arctic charr (*Salvelinus alpinus*) were present in the compartment to the left of the divider.

**Table 2.** Mean standard length* of *Gasterosteus aculeatus* for each shoaling density replicate (*N* = 22 for all populations)

<table>
<thead>
<tr>
<th>Replicate 1</th>
<th>TLAVA</th>
<th>TSOFT</th>
<th>FLAVA</th>
<th>FSOFT</th>
<th>SAUR</th>
<th>STOKK</th>
<th>HVASS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>48.2</td>
<td>51.5</td>
<td>69.1</td>
<td>61.3</td>
<td>47.0</td>
<td>52.5</td>
<td>60.1</td>
</tr>
<tr>
<td>Standard error</td>
<td>1.44</td>
<td>0.62</td>
<td>1.19</td>
<td>1.20</td>
<td>0.87</td>
<td>0.49</td>
<td>1.24</td>
</tr>
<tr>
<td>Replicate 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length (mm)</td>
<td>40.5</td>
<td>47.4</td>
<td>57.7</td>
<td>56.6</td>
<td>41.4</td>
<td>51.4</td>
<td>54.5</td>
</tr>
<tr>
<td>Standard error</td>
<td>1.50</td>
<td>0.75</td>
<td>0.82</td>
<td>1.25</td>
<td>0.83</td>
<td>0.62</td>
<td>0.87</td>
</tr>
</tbody>
</table>

*Mean standard length measured to nearest 0.5 mm. Site abbreviations are defined in Table 1.
gender effects would be minimal. Furthermore, because of the monomorphic external appearance of the two genders when not in breeding colours, a fish's gender could not be determined without dissection.

In initial trials predators were absent, and *G. aculeatus* were transferred directly from the holding tanks to the test aquarium. They were allowed 15 min to acclimatize to the tank before recording began. Two video cameras, one directed at the front of the tank and the other at the mirror angled above the tank, were used to record the behaviour and position of the group for 20 min. The fish were returned to their holding tanks at the end of the 20-min trial.

Twenty-four hours after each initial shoaling trial (with predators absent), the same groups of *G. aculeatus* were retested in a second trial, in which the three predators were positioned in the smaller sector of the aquarium prior to the release of *G. aculeatus* into the larger section. We used live predators because previous studies have shown that fish habituate more slowly to real predators than they do to models (Pitcher, 1992). Because the predators were already present and *G. aculeatus* were familiar with the tank, the procedure for introducing *G. aculeatus* to the test tank was altered in this second trial. They were placed in a Plexiglas box, with a bottom of mesh screening, and held in the water at the far side of the tank for 1 min prior to release in the centre of the tank. This allowed them to recover from the transfer and adjust to the lighting without direct observation of the predators. They showed no response to the presence of the predators prior to release from the box.

The videotapes were later analysed for shoaling density by pausing the tape every 30 s throughout the first 15 min of each predator-absent and predator-present trial and recording the three-dimensional position of each *G. aculeatus* in the grid pattern (i.e. instantaneous scan sampling). The video recordings had to be forwarded second-by-second and reversed regularly to ensure the accurate position of all 22 fish in three-dimensional space. Only the first 15 min of the trials were used due to the time required to complete this task, yielding 30 instantaneous measures of shoaling density for each of the two replicates.

A 'shoal' is a group of fish that remain together due to social attraction (Pitcher & Parrish, 1993). A 'school' refers to a synchronized and cohesive group that is orientated and moving together in the same direction (polarized), making it a subcategory in the broader term of shoaling (Pitcher & Parrish, 1993). In this paper, shoaling density/cohesion was examined using a grid to determine the spatial organization of a group of *G. aculeatus*; schooling was examined separately as antipredator behaviour that occurs in the presence of a predation threat.

### Observations of Predator Inspection and Avoidance Behaviour

The video recordings from the predator-present trials were analysed further for inspection and avoidance behaviour related to the presence of predators. Inspection, schooling, fleeing and startle responses were recorded from the front view (behaviour categories are defined in Table 3), using The Observer (Noldus, Netherlands, Version 3) behaviour recording software. States and events were recorded for the entire duration of the 20-min trial. Each trial analysis required that the recording be viewed several times for each event or state to be recorded accurately. Because of the extensive time required, and the observation that both replicates from each of the populations showed very similar behaviour, only one trial was analysed for each *G. aculeatus* population.

Movements of the predatory fish were recorded throughout the trials. Reactions to predator movements differed between groups and no direct correlation was found. For instance, a charge at the divider by a predator would elicit startle and flee responses in one group of *G. aculeatus* but no reaction in another. Furthermore, some groups showed frequent startle and flee responses when predators were stationary or facing away from them.

### Table 3. Ethogram for antipredator behaviour of *Gasterosteus aculeatus*

<table>
<thead>
<tr>
<th>States</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inspection</td>
<td>One or more <em>G. aculeatus</em> leave the main shoal and slowly approach the predator, remain still in the water column facing the predator, and later return to the shoal; recorded from time inspecting individuals approach within 50 cm of the predator until they turn to return to the main shoal; recorded for groups of 1, 2, 3, 4, 5, 6, 7–9, and ≥10 <em>G. aculeatus</em></td>
</tr>
<tr>
<td>Schooling</td>
<td>Groups of three or more <em>G. aculeatus</em> that are swimming in a synchronized and polarized group; recorded for groups of 3, 4, 5, 6–10, 11–15, and ≥16 <em>G. aculeatus</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Events</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Startle response</td>
<td>Flexing of the body laterally (C-shape) combined with a quick release which results in a fast movement of the <em>G. aculeatus</em> away from a perceived threat; sometimes followed by fleeing; recorded for groups of 1, 2, 3, 4, 5 and ≥5 <em>G. aculeatus</em></td>
</tr>
<tr>
<td>Flee response</td>
<td>A sudden swimming dash in a direction away from a predator or a perceived threat; recorded for groups of 1, 2, 3, 4, 5, 6, and ≥6 <em>G. aculeatus</em></td>
</tr>
</tbody>
</table>
STATISTICAL ANALYSIS OF SHOALING DENSITY

To determine shoaling density, frequency histograms for both predator-absent and predator-present trials were created to show the frequency in which groups of 1–22 *G. aculeatus* were recorded in each three-dimensional cube (see Appendix 1, Doucette, 2001). No differences in shoaling density were found between the two replicates for each of the seven populations (Mann–Whitney (M–W) U-tests: *N* = 22, *P* > 0.05). Therefore, the data for the two replicates were pooled to create a two-way table of responses for the frequency of groups of ≤5 and >5 *G. aculeatus* occurring within a single grid cube, and the results were plotted as bar graphs (Fig. 2; Koopmans, 1987). The presence of >5 *G. aculeatus* within a grid cube was indicative of a tightly formed shoal.

To further examine the shoaling density, an ‘index of cohesion’ was calculated (Seghers, 1974). At each 30-s observation, the maximum density for any of the 96 grids was recorded. The index is the mean maximum density for 30 consecutive observations in each of two separate replicates. It has a theoretical minimum of one (all *G. aculeatus* found singly) and a maximum of 22 (entire group in the area of one cube). Kruskal–Wallis (K–W) and M–W U-tests were used to compare the differences among populations for mean maximum shoal density (Sokal & Rohlf, 1994).

STATISTICAL ANALYSIS OF PREDATOR INSPECTION AND AVOIDANCE BEHAVIOUR

Inspection and schooling data were examined to determine: (1) the intensity of the respective behaviour by calculating the total duration for which an inspection unit or school existed during the trials, and (2) the cost to the individual *G. aculeatus* by calculating the duration of time that an average individual spent in an inspection unit or school. *G. aculeatus* were classified as an inspecting unit as long as they were all inspecting and responding to the predators synchronously. They were classified as schooling when they were moving as in synchronized and polarized formation. The total duration of inspection and schooling behaviour was calculated and compared for the seven *G. aculeatus* groups (K–W test) and specifically for the sympatric groups within lakes (M–W U-test). To determine the cost of inspection and schooling for an average individual *G. aculeatus*, the number of fish demonstrating that behaviour was multiplied by the duration of the behaviour. For the purposes of statistical analysis, the duration of a behaviour occurring within each minute of the 20-min trial was treated as a subsequent observation or bout (Lehner, 1996). The total number of startle and flee responses was calculated for each group and compared using K–W and M–W U-tests.

RESULTS

SHOALING DENSITY AND COHESION

*Gasterosteus aculeatus* from both TSOFT and HVASS sites shoaled in groups >5 more in comparison with the other sites when predators were absent (Fig. 2A). The combined mean of all groups revealed that shoals containing >5 *G. aculeatus* were formed only 23% of the time. However, the TSOFT group formed shoals of ≥10 49% of the time and the HVASS group formed these larger shoals 71% of the time. *G. aculeatus* from HVASS showed the highest shoaling affinity, with 53% of HVASS *G. aculeatus* recorded in shoals of ≥10 and only 8% of the fish recorded singly. The TSOFT group formed shoals of ≥10 *G. aculeatus* 34% of the time. In contrast, FSOFT and SAUR groups
rarely formed shoals of >5 *G. aculeatus*, and the FLAVA group never formed such shoals (Fig. 2A).

When predators were present there were marked changes in shoaling density. *G. aculeatus* from TSOFT increased their shoaling density, forming shoals >5 64% of the time (Fig. 2B) and, forming shoals >10 49% of the time. TSOFT *G. aculeatus* were found singly for only 10% of the predation trial. Similarly, FLAVA, FSOFT and SAUR groups all increased the number of large shoals formed in the presence of predators (Fig. 2B). Contrary to what one might predict, the HVASS group decreased their shoaling density and tended to form shoals of ≤5 *G. aculeatus* 81% of the time vs. 29% when predators were absent. TLAVA and STOKK groups also decreased the number of large shoals formed (13% vs. 19% and 3% vs. 20%, respectively) when predators were present.

Comparisons between the two replicate trials for both predators absent and predators present revealed that in some cases the second group tested, consisting of slightly smaller fish, showed higher shoaling cohesion (Table 4). Juveniles of most fish species are known to form cohesive shoals (Pitcher & Parrish, 1993). While all *G. aculeatus* tested in these experiments were adults, it is possible that the smaller fish (second trials) were slightly younger and less experienced. These fish would achieve greater security in larger and more compact shoals (Seghers, 1974; Magurran, 1990). The only groups to show significantly greater shoaling cohesion for the second trial with and without predators present were FSOFT and SAUR. Generally, shoaling cohesion for both of these groups was very low, thus this difference between trials had little impact on the overall results.

There was a highly significant difference in the mean maximum shoal density among all groups both when predators were absent (K–W test: $H_0 = 275.8$, $P < 0.00001$) and when predators were present (K–W test: $H_0 = 191.1$, $P < 0.00001$). As expected, the index of cohesion revealed similar trends as in the analysis of shoals (Fig. 3). When predators were present a significant increase in mean maximum density was seen in three of the groups: TSOFT (M–W *U*-test: $U = 2359.5$, $N_1 = N_2 = 60$, $P < 0.005$), FLAVA ($U = 2838.5$, $N_1 = N_2 = 60$, $P < 0.00001$), and FSOFT ($U = 2975$, $N_1 = N_2 = 60$, $P < 0.00001$). However, the mean maximum density of HVASS shoals decreased from 13.3 to 6.1 *G. aculeatus* when predators were present (M–W *U*-test: $U = 377$, $N_1 = N_2 = 60$, $P < 0.00001$).

**Predator inspection and avoidance behaviour**

We concluded that in the HVASS and TLAVA groups it was the high incidence of inspection behaviour that led to decreased shoal size when predators were present. The continuous movement of one or more *G. aculeatus* away from the main shoal to inspect caused a decrease in shoal density. All groups had one or more *G. aculeatus* inspecting during at least 76% of the total trial duration (Table 5). There was a significant difference between groups for the total duration of time that an inspection unit was present (K–W: $H_0 = 29.99$, $P < 0.00005$; Table 5). The TLAVA and SAUR groups had longer inspection durations than did the other groups (Dunn’s multiple comparison, $P < 0.05$). When the duration of units of ≤5 *G. aculeatus* and >5 *G. aculeatus* inspecting were examined separately, the differences between groups was even greater (K–W: $H_0 = 41.04$, $P < 0.00001$ and $H_0 = 41.88$, $P < 0.00001$, respectively). These differences were caused by the tendency for TLAVA *G. aculeatus* to inspect in much larger units than did all other populations (Table 5). The number of inspection events per trial for TLAVA *G. aculeatus* was less than it was in the other groups, indicating that these

| Site abbreviations are defined in Table 1. | © 2004 The Linnean Society of London, Biological Journal of the Linnean Society, 2004, 82, 189–203 |

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**Table 4.** Mean maximum shoal density (cohesion) and results of Mann–Whitney *U*-tests for seven *G. aculeatus* populations comparing the two replicate trials ($N_1 = N_2 = 30$ for all populations)

<table>
<thead>
<tr>
<th></th>
<th>TLAVA</th>
<th>TSOFT</th>
<th>FLAVA</th>
<th>FSOFT</th>
<th>SAUR</th>
<th>STOKK</th>
<th>HVASS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Predators absent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Replicate 1</td>
<td>3.10</td>
<td>10.13</td>
<td>2.13</td>
<td>2.30</td>
<td>2.50</td>
<td>3.03</td>
<td>13.73</td>
</tr>
<tr>
<td>Mean Replicate 2</td>
<td>7.87</td>
<td>10.93</td>
<td>2.03</td>
<td>3.30</td>
<td>3.70</td>
<td>3.13</td>
<td>12.87</td>
</tr>
<tr>
<td><em>U</em>-value</td>
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<td>500</td>
<td>420</td>
<td>661</td>
<td>671</td>
<td>492</td>
<td>399.5</td>
</tr>
<tr>
<td><em>P</em>-value &lt;0.05</td>
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<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Predators present</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Replicate 1</td>
<td>4.93</td>
<td>14.60</td>
<td>3.20</td>
<td>3.47</td>
<td>3.20</td>
<td>3.83</td>
<td>5.13</td>
</tr>
<tr>
<td>Mean Replicate 2</td>
<td>5.50</td>
<td>10.90</td>
<td>4.23</td>
<td>7.40</td>
<td>3.97</td>
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<td>7.10</td>
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<tr>
<td><em>U</em>-value</td>
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<td>178.5</td>
<td>518</td>
<td>862</td>
<td>594.5</td>
<td>419</td>
<td>603.5</td>
</tr>
<tr>
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<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
large units continued inspecting for long periods of time before returning to the main shoal. There were clear differences in inspection duration between the two groups from Thingvellavatn. The total time that TLAVA G. aculeatus inspected was significantly greater (M–W U-test: $U = 203, N_1 = N_2 = 20, P < 0.05$) and the time that TLAVA G. aculeatus inspected in units >5 was longer than it was for the TSOFT G. aculeatus (M–W U-test: $U = 364, N_1 = N_2 = 20, P < 0.00001$). Total inspection duration did not differ between the two groups in Frostastaðavatn (M–W U-test: $U = 145, N_1 = N_2 = 20, P = 0.14$).

Based on the duration of inspection and the number of G. aculeatus in each inspection unit, the duration that an individual inspected was calculated (Pitcher, 1992). There were significant differences among groups in the total duration that each G. aculeatus performed inspection behaviour (K–W: $H_a = 50.19, P < 0.00001$; Fig. 4). These differences were due mainly to the much longer duration of individual inspection by the TLAVA G. aculeatus (Dunn’s multiple comparison test: $P < 0.05$). An individual in the TLAVA population spent an average of 64% of its time inspecting compared with a maximum of 25% (SAUR) in other groups, and it was almost always in a group of >5 G. aculeatus. There was a large difference in total individual inspection duration between the two groups in Thingvellavatn (M–W U-test: $U = 400.0, N_1 = N_2 = 20, P < 0.00001$), but no significant differences between the two groups in Frostastaðavatn (M–W U-test: $U = 177.0, N_1 = N_2 = 20, P = 0.54$).

The duration for which a school was present during the predation trials varied greatly among the groups (K–W: $H_a = 32.79, P < 0.00001$; Table 5). As with shoaling behaviour, HVASS G. aculeatus showed the greatest duration of schooling (41%) followed by TSOFT G. aculeatus (24%). The two marine groups (STOKK and HVASS) and TSOFT G. aculeatus spent significantly more time schooling than did the other groups (Dunn’s multiple comparison test: $P < 0.05$). The marine populations also tended to form larger schools both in terms of size at first school and most frequent school size (Table 5). SAUR G. aculeatus only schooled for 1% of the total trial time. There were no significant differences in total school duration between the two groups in Thingvellavatn (M–W U-test: $U = 136.5, N_1 = N_2 = 20, P = 0.09$) or in Frostastaðavatn (M–W U-test: $U = 210, N_1 = N_2 = 20, P = 0.80$). However, the TSOFT group formed more small schools of ≤5 G. aculeatus than did the TLAVA group (M–W U-test: $U = 90.5, N_1 = N_2 = 20, P < 0.005$). Individuals from the two marine populations spent more time schooling than did individuals from other groups (K–W: $H_a = 31.66, P < 0.00005$; Fig. 5). In most groups, individuals tended to be in schools of >5 G. aculeatus. However, TSOFT G. aculeatus formed small schools of ≤5 more often than did the other groups (K–W: $H_a = 43.67, P < 0.00001$; Dunn’s multiple comparison test: $P < 0.05$). There were no significant differences in the total duration for which an individual schooled between the sympatric groups in Thingvellavatn (M–W U-test: $U = 151.5, N_1 = N_2 = 20, P = 0.19$) nor between the groups in Frostastaðavatn (M–W U-test: $U = 216, N_1 = N_2 = 20, P = 0.67$).

Differences among groups in numbers of startle and flee responses was highly significant (K–W: $H_a = 43.28, P < 0.00001$ and $H_a = 60.70, P < 0.00001$, respectively). The TLAVA G. aculeatus executed a

Figure 3. Index of cohesion showing the mean maximum shoal density for seven groups of Gasterosteus aculeatus. Boxes represent the means of two replicates and 60 instantaneous samples per group. Error bars represent ±95% confidence intervals and dashes represent the maximum and minimum values of maximum shoal density for each group. (A) Predators absent. (B) Predators present. Site abbreviations are defined in Table 1.
larger number of startle and flee responses than did all other groups (Dunn’s multiple comparison test: \( P < 0.05; \) Fig. 6). There was no significant difference in the number of startle responses between the two groups in Thingvallavatn (M–W \( U \)-test: \( U = 146, N_1 = N_2 = 20, P = 0.15 \)) but there was a clear difference in the number of fleeing manoeuvres (M–W \( U \)-test: \( U = 290, N_1 = N_2 = 20, P < 0.05 \)) in Frostastaðavatn.
The results of this study show clear differences in antipredator behaviour, including shoaling, schooling, and startle and flee responses, between *G. aculeatus* populations residing in different habitats. These differences appear to relate to the apparent risk of predation. *G. aculeatus* from Thingvallavatn, where the risk of predation is very high (Malmquist *et al.*, 1992), showed the most vigorous antipredator responses. The groups from Thingvallavatn also exhibit the greatest morphological differences compared with *G. aculeatus* pairs in other volcanic Icelandic lakes (Kristjánsson, 2001; Kristjánsson *et al.*, 2002a) and show clear differences in other behaviour (Doucette, 2001). In contrast, in Frostastaðavatn, where the risk of predation may be lower, morphological (Kristjánsson *et al.*, 2002a) and behavioural divergence (Doucette, 2001) of *G. aculeatus* is less. Despite the presence of piscine predators, neither Frostastaðavatn group showed well-developed antipredator responses, and the shoaling cohesion of both groups was very low. *G. aculeatus* from Sauravatn, where no piscine predators exist, showed almost no antipredator behaviour. Marine populations relied almost entirely on shoaling and schooling to deter predators. Freshwater populations appear to have lost some affinity for forming the dense shoals or schools seen in marine populations, perhaps in favour of seeking refuge when confronted by a potential predator. Results suggest that the risk of predation may be an important contributing factor in promoting ecological divergence and maintaining separate forms. Under the right circumstances, predation pressure could force prey to compete for predator-free resource habitats and lead to sympatric speciation.

One morph of *Salv. alpinus* in Thingvallavatn is specialized to feed on *G. aculeatus* (Sandlund *et al.*, 1987; Malmquist *et al.*, 1992). Based on diet analysis of *Salv. alpinus* in the littoral lava habitat, between 35% and 100% of the stomachs of the large limnetic piscivore morph contained *G. aculeatus* (diet varied by season). Between 65% and 100% of the ash-free dry weight (AFDW) of the large limnetic morph’s diet consisted of *G. aculeatus*. In the profundal and littoral *Nitella* (soft-bottom) habitats 44–100% of the large limnetic morph stomachs contained *G. aculeatus* (AFDW = 36–88%). The present habitat structure of the lake was already in place shortly after the ice retreated from the area around 10 000 years ago (Sæmundsson, 1992). *Salv. alpinus*, *Salmo trutta* and *G. aculeatus* most probably colonized the lake quickly after this. The presence of a specialist piscivorous *Salv. alpinus* in Thingvallavatn (Sandlund *et al.*, 1987; Malmquist *et al.*, 1992) suggests that predation pressure on *G. aculeatus* has been intense for thousands of years.

While both Thingvallavatn groups showed a clear reaction to the predators, their responses were vastly different. The TSOFT population in Thingvallavatn shoaled cohesively, a suitable response in a comparatively open, vegetated environment. It is well known that small prey fish reduce individual risk of predation while shoaling and will form larger shoals in high-risk localities, even in the absence of direct threat, as vigilance increases with shoal size (Seghers, 1974; Magurran & Seghers, 1994a; Magurran, 1999). Typically, when a potential underwater predator is detected, shoals become more compact and cohesive (Pitcher & Parrish, 1993), as was the case for the TSOFT population, which formed polarized schools for 24% of the duration of the trial. In contrast, the TLAVA *G. aculeatus* from Thingvallavatn showed only moderate shoaling and schooling behaviour, likely due to the availability of sub-benthic spaces in their habitat that provide safe cover from large predatory fish. The bottom of the Vatnsvik inlet, where the TLAVA *G. aculeatus* were collected, is composed of broken lava rock with sub-benthic spaces and rifts, which may reach depths of more than 10 m (Sæmundsson, 1992). The spaces between and under rocks provide small fish with a protected environment (Snorrason *et al.*, 1994). *G. aculeatus* are rarely observed in the open water above the lava rubble. The intense monitoring and inspection behaviour may enable the TLAVA *G. aculeatus* to evade predation by early detection of motivated predators. The benefit of inspection is that it provides continually updated information about predator identity and likelihood of attack (Pitcher, 1992). This reduces the risk to all individuals by allowing attack anticipation. When the perceived risk is high, risk dilution or ‘safety in numbers’ is achieved by

![Figure 6.](image-url)
forming larger inspection units (Magurran, 1990; Pitcher, 1992; Magurran & Seghers, 1994a). In this study, inspection units of TLAVA *G. aculeatus* often approached the maximum shoal size of 22 (see Magurran & Pitcher, 1987) whereas the predominant inspection unit size for *G. aculeatus* from all other sites was one or two. The TLAVA *G. aculeatus* coordinated their lengthy inspection bouts with the frequent use of startle and flee responses. Despite the variability in antipredator behaviour in the two groups from Thingvallavatn, both showed appropriate responses to a high-risk predatory habitat.

The antipredator responses observed for both groups of *G. aculeatus* in Frostastaðavatn were minimal. Both groups showed low levels of shoaling and schooling behaviour. Based on lake structure, FSOFT *G. aculeatus* would be expected to show a high degree of schooling and shoaling behaviour, similar to the intense schooling behaviour of TSOFT *G. aculeatus*. Only the relatively high number of startle and flee responses of the FSOFT *G. aculeatus* suggests that these fish are from a lake with a dense population of potential piscine predators. In Frostastaðavatn, *G. aculeatus* have most likely evolved without threat from piscine predators until *Salv. alpinus* and *Salmo trutta* were introduced about 30 years ago (B. Jónsson, pers. comm.). The time passed since the introduction of *Salv. alpinus* equals approximately 15 *G. aculeatus* generations, which is probably too short to cause a significant evolutionary response. Furthermore, *Salv. alpinus* in Frostastaðavatn may not be predisposed to feed on *G. aculeatus*. Stomach content analysis indicated that only 4% of their diet was composed of *G. aculeatus* (B. Jónsson, unpubl. data).

The lack of predation threat in Sauravatn has led to a paucity of antipredator behaviour in SAUR *G. aculeatus*. Shoaling cohesion was extremely low and increased only minimally when predators were present. These fish showed almost no schooling behaviour, and inspection was equal to or less than that in all other groups. Startle and flee responses were also rare. The presence of piscine predators during the experiments did not elicit behavioural responses considered to reduce predation risk. The inspection behaviour that was observed was perhaps due more to curiosity than fear. This population of *G. aculeatus* represents an excellent control group for antipredator behaviour, enforcing the prediction that low natural antipredator behaviour would exist in *G. aculeatus* from a lake with no risk of piscine predation.

The marine *G. aculeatus* populations were selected as examples of the ancestral form of *G. aculeatus* in Iceland for comparison with the evolved freshwater populations (Bell & Foster, 1994a). The marine populations showed dense shoaling and schooling as their main means of antipredator defence. Freshwater pop-

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Ecological stability, discreteness and heterogeneity in niches, and low fish-species diversity are seen as relevant features promoting phenotypic divergence (Maynard Smith, 1966; Malmquist et al., 1992; Skúlason et al., 1999; Snorrason & Skúlason, 2004). Both Thingvallavatn and Frostastaðavatn are in areas of permeable bedrock and each lake is fed primarily through underground springs (Adalsteinsson et al., 1992; B. Jónsson, pers. comm.). Thus, the waters in both lakes are rich in minerals, and are generally stable and productive (Jónasson, Adalsteinsson & Jónsson, 1992.). Both lakes contain only three species of fish, hence there is low interspecific competition, and have relatively similar lava and soft-bottom habitats. So why is there not the same level of morphological (Kristjánsson et al., 2002a) and behavioural (Doucette, 2001) divergence between G. aculeatus occupying the two diverse habitats in each lake? When considering the variable degree of divergence of sympatric groups of G. aculeatus in Thingvallavatn and Frostastaðavatn we must examine several ecological factors that differ between the lakes and which may influence the probability of morph divergence: (i) the lake size, (ii) the evolutionary time available for habitat-specific divergence, and (iii) the level of predation and the proximity of alternative G. aculeatus habitats.

Thingvallavatn has an area of 83 km² and a mean depth of 34 m (Adalsteinsson et al., 1992) whereas Frostastaðavatn is only 2.55 km² and has a mean depth of 6.5 m (H. Malmquist & G. Gudbergsson, pers. comm.). However, other Icelandic lakes of very small size have been found to have two different morphs of Salv. alpinus (Galtaböl = 1.4 km², Gislason et al., 1999; Vatnshlídarvatn = 0.7 km², Jónsson & Skúlason, 2000). Thus, it is possible for discrete phenotypes to evolve and maintain genetic distinction in lakes of a very limited size (see also Schilewan, Tautz & Pääbo, 1994), and the small size of Frostastaðavatn should not constrain the evolution of different morphs.

Thingvallavatn was probably isolated from external invasions of fish as early as 9130 ± 260 years ago when the outlet was dammed by lava (Sæmundsson, 1992). The age and history of Frostastaðavatn is far less certain. What is known, though, is that the lava habitat in the lake was formed after lava flowed into the lake in the year 1477 AD. This means that the scope for habitat-specific divergence of the G. aculeatus in Frostastaðavatn is at the most about 250 generations, i.e. at least ten times shorter than it is in Thingvallavatn. Evolutionary responses to changes in predatory threat can certainly be significant in such a short time (e.g. Magurran, 1999; Kristjánsson, Skúlason & Noakes, 2002b). This, and the fact that habitat morphs in Icelandic G. aculeatus seem to be related (Kristjánsson, 2001; Kristjánsson et al., 2002a), suggest that we should be observing more distinct habitat morphs in Frostastaðavatn. The fact that we do not may stem from the lack of predation threat.

The origin of the introduced piscine predators in Frostastaðavatn is unknown; therefore we have no information on the level of experience of G. aculeatus in piscivorous predation. However, as mentioned earlier, stomach content analysis of Salv. alpinus in Frostastaðavatn from 1994 showed that only 4% of their diet consisted of G. aculeatus (B. Jónsson, unpubl. data). In contrast, up to 99% (AFDW) of the diet of the large limnetic morph of Salv. alpinus in Thingvallavatn was composed of G. aculeatus (Malmquist et al., 1992). We can reasonably conclude that the risk of predation is lower in Frostastaðavatn than it is in Thingvallavatn. The difference in the degree of antipredator behaviour between G. aculeatus in these two lakes clearly supports this conclusion.

A small fish, such as G. aculeatus, that is exposed to intense predation from piscivorous fish species will be driven to seek refugia and food in sheltered habitats (Werner et al., 1983; Sandlund et al., 1992). In lakes where more than one protective habitat is offered, the apparent competition to seek shelter can quickly promote local adaptation to the respective resources (Holt, 1977). In Thingvallavatn, there is a great expanse of open water, densely populated by piscivorous Salv. alpinus and some Salmo trutta, separating the protective vegetated Nitella stands and the littoral lava habitat. G. aculeatus have not been found in this open pelagic zone of Thingvallavatn (Sandlund et al., 1992; B. K. Kristjánsson & L. I. Doucette, unpubl. data). The importance of macrophyte vegetation in reducing predation on small fish has been shown (Werner et al., 1983), and there is likely competition for the sheltered habitats offered by both the Nitella and lava habitats and avoidance of the open water. Jakobsen, Johnsen & Larsson (1987) found that introduction of salmonid predators induced a habitat shift in G. aculeatus from open to sheltered areas in a small lake in western Norway. Thus, the high risk of predation in Thingvallavatn could be preventing the G. aculeatus occupying the two protective habitats from intermixing and interbreeding, thereby accelerating the divergence process. In Frostastaðavatn, the overall risk of predation appears to be lower and the two protective habitats are in closer proximity to each other. G. aculeatus occupying the two habitats may be able to move between them with a lower risk of predation. The result may be a lower degree of isolation between the two groups. This may reduce the degree of specialization of the Frostastaðavatn G. aculeatus for their respective habitats and may greatly reduce the likelihood, or at least the rate of, sympatric diver-
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Perhaps the morphs in Thingvallavatn and Frostastaðavatn are at ‘different stages of segregation’, with the segregation more advanced in Thingvallavatn.

In view of the above ecological considerations and our behavioural data, we emphasize that a high risk of predation in Thingvallavatn may have played a major role in the evolution of unique and discrete foraging behaviour and morphology in this lake, as the two forms specialized for two different habitats with reduced predation risk. Conversely, the lack of predation risk, and the closer proximity of protective habitats to each other, may have contributed to the low divergence seen in Frostastaðavatn.

We conclude that risk of predation may play an important role in sympatric divergence of fish, especially for species in which adult body size is small and these smaller prey fish are forced to seek refugia and food in sheltered habitats. The combined effects of ecological factors and predation pressure on morph formation and sympatric species divergence have very likely been underestimated in the past. It would be interesting to re-examine some studies on resource polymorphisms and species divergence in species of small fish, such as cichlids (Meyer, 1993), considering the potential impact of predation risk and the relative locations of alternative habitats on the evolution of polymorphisms and speciation.

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REFERENCES


Skulason S, Snorrasson SS, Ota D, Noakes DLG. 1993. Genetically based differences in foraging behaviour among...


