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Oil exposure disrupts early life-history stages of coral reef fishes via behavioural impairments

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Global demand for energy and oil-based products is progressively introducing petrogenic polycyclic aromatic hydrocarbons (PAHs) into sensitive marine environments, primarily from fossil-fuel exploration, transport, and urban and industrial runoff. These toxic pollutants are found worldwide, yet the long-term ecological effects on coral reef ecosystems are unknown. Here, we demonstrate that oil exposure spanning PAH concentrations that are environmentally relevant for many coastal marine ecosystems ($\leq$5.7 μg l^{-1}), including parts of the Great Barrier Reef, Red Sea, Asia and the Caribbean, causes elevated mortality and stunted growth rates in six species of pre-settlement coral reef fishes, spanning two evolutionarily distinct families (Pomacentridae and Lethrinidae). Furthermore, oil exposure alters habitat settlement and antipredator behaviours, causing reduced sheltering, shoaling and increased risk taking, all of which exacerbate predator-induced mortality during recruitment. These results suggest a previously unknown path, whereby oil and PAH exposure impair higher-order cognitive processing and behaviours necessary for the successful settlement and survival of larval fishes. This emphasizes the risks associated with industrial activities within at-risk ecosystems.

Each year, over six million metric tons of petroleum products are estimated to enter global oceans from anthropogenic sources such as industrial discharge, urban run-off and shipping operations[1,2]. Additionally, oil exploration and transport have resulted in more than 340 major marine oil spills in the past 40 years, releasing over 3,900 million metric tons of crude oil into the environment[3]. Fresh petroleum associated with the oil exploration and production industry is generally classified as heavy crude oil, which contains approximately 0.6–6.0% polycyclic aromatic hydrocarbon (PAH) compounds by weight[4,5]. These petrogenic compounds are known to be toxic to marine life (for example, refs [6–8]) and highly carcinogenic, mutagenic and teratogenic for marine biota at concentrations as low as 1.2 μg l^{-1} (refs [4,9,10]).

Due to their many sources, capacity for dissolution in the water column, high level of toxicity and slow breakdown, petrogenic PAHs are nearly ubiquitous environmental contaminants classified as persistent organic pollutants[2] that can be found in coastal waters and sediments worldwide[1,2,11–14]. Typical PAH concentrations in the Americas, Europe, the Middle East, Asia and Australia range from less than 0.1 to over 600 μg l^{-1} (refs [1,2,11–14]), but may reach concentrations as high as 9,000 μg l^{-1} in heavily polluted sites[11]. Near urban centres, in areas with large industrial or shipping operations, and near oil exploration sites, PAH concentrations are at the highest levels[11–13].

The demand for petroleum products is increasingly driving the oil exploration and production industry to encroach on remote, pristine ecosystems in the Arctic and tropical coral reefs[15–17]. At present, more than 400 million people directly depend on tropical coral reefs for survival[18], and this ecosystem is estimated to provide over US$30 billion in annual revenue from fisheries and tourism globally[18]. However, tropical coral reefs are also some of the fastest degrading and disappearing habitats on the planet, primarily due to anthropogenic activities[19,20]. These include overexploitation (for example, fish, coral and shellfish harvesting), global climate change, and poor water quality and pollutants from land-based effluent and heavy industry[19,20]. Over the past 35 years, an estimated 19% of the world's coral reefs has completely disappeared. A further 15% is expected to disappear within the next 10–20 years, and more than 35% is under severe threat of disappearing within 40 years[19,20]. In 2016 alone, more than 35% of corals on the northern Great Barrier Reef are estimated to have died following the worst bleaching event ever recorded[21]. Despite these alarming developments, many governments continue to push for increased industrial activities in reef habitats aimed at short-term gains[15,16]. There is increasing concern that additional stress from human activities, including petroleum pollution, may be eroding the resilience of the remaining reef ecosystems and escalating their decline[8,15]. Due to the distribution and persistence of petrogenic pollutants, exposure of reef organisms is particularly likely near sites of industrial activity; however, the mechanisms by which these pollutants manifest have not been fully elucidated.

The early life-history stages of reef organisms, including embryos and larvae, are considered to be the primary conduit for the replenishment and abundance of keystone species to reef ecosystems[22]. In fish, early life stages typically experience extreme mortality rates, exceeding 90% within the first few weeks of life[22,23]. During recruitment, individuals are under strong selection for traits related to finding a suitable habitat, predator avoidance and growth[24]. Increased mortality during this sensitive stage may have unforeseen downstream ecological consequences, including a reduced abundance of keystone species, such as algal herbivores, which help to maintain a coral-dominated stage[19,20]. This would ultimately erode the capacity of the ecosystem to resist and recover from perturbation[19,20]. However, organisms are also particularly vulnerable to pollutants during this period due to their less-developed homeostatic mechanisms, immune responses and organ systems. Studies on the early life stages of non-coral reef fish species have consistently demonstrated that sublethal PAH exposure can cause cardiac, spinal and craniofacial deformities[7,10,25]. The pronounced pericardial edema and compromised cardiomyocyte function resulting from

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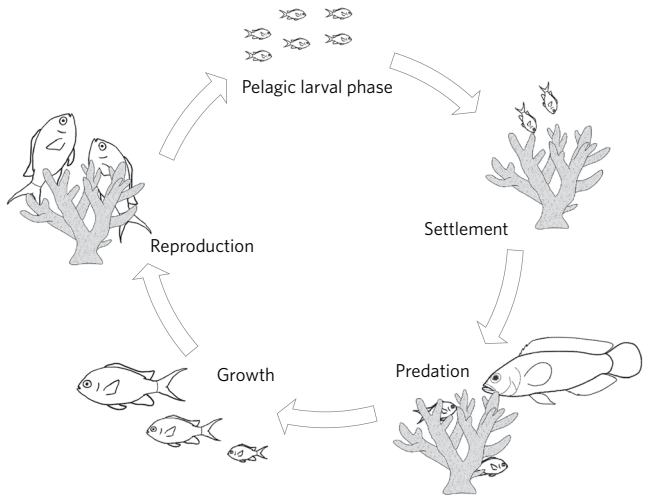

Figure 1 | Schematic life cycle of coral reef fishes. This study examined the impact of oil pollution exposure on the first four events of this life cycle, including the PLP, settlement, predation and growth.

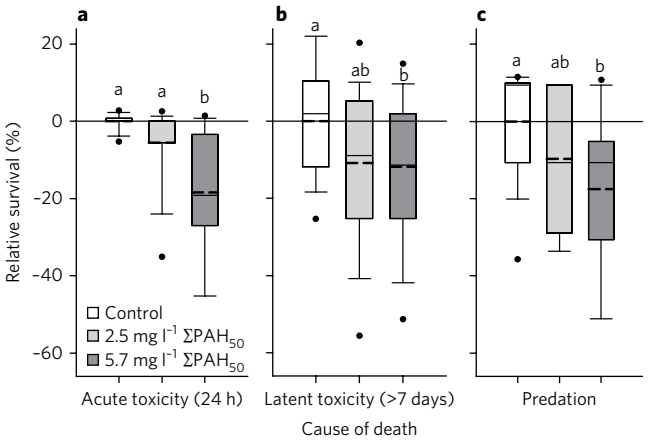

Figure 2 | Excess mortality of crude oil HEWAF-exposed larval coral reef fishes. a, Relative survival of *C. atripectoralis*, *P. amboinensis*, *P. bankieri*, *P. chrysurus*, *P. moluccensis* and *Lethrinidae* species due to acute toxicity after 24 h. **b**, Relative survival of *P. amboinensis*, *P. chrysurus* and *P. moluccensis* due to latent toxicity after more than seven days. **c**, Relative survival of *P. amboinensis* and *P. moluccensis* due to predation. Oil exposures are expressed as ΣPAH_{50} concentrations. Significant differences are marked above each column by letters. Dots represent the 5th and 95th percentiles (bottom and top, respectively); error bars represent the 10th and 90th percentiles; box edges represent the 25th and 75th percentiles. Within the boxes, dashed lines show means and solid lines show medians.

embryonic or juvenile PAH exposure[26] is known to reduce cardiac output and has been implicated in increased metabolic stress and reduced swimming performance in later life[6,9,25]. PAH exposure can also alter gene expression relating to neurological systems in the early life stages of fish[27], with unknown consequences for sensory systems, brain function and behaviour. Thus, these sublethal effects could seriously compromise the ecology and long-term survival of coral reef fishes—a group of organisms whose sensitivity to oil toxicity is yet to be thoroughly investigated, particularly during the challenging recruitment phase of their life cycle when they are most vulnerable.

To understand the ecological effects of petrogenic pollution on coral reef fish recruitment, we examined the effects of crude oil exposure on the success of settlement, growth and survival of fish larvae during the first few weeks of life. Importantly, this period encompasses four out of five major life-history challenges in most reef fishes: (1) a post-hatch 16–28-day pre-settlement pelagic larval phase (PLP); (2) a settlement stage in which to find suitable reef habitat; (3) rapid post-settlement learning to identify and avoid predators; and (4) rapid growth to establish territory and secure shelter, before ultimately reaching (5) reproductive maturity (Fig. 1; ref. [28]). We used high-energy water accommodated fractions (HEWAFs) of naturally weathered crude oil to expose fish larvae to environmentally relevant concentrations of PAHs (0, 2.5 and 5.7 $\mu g\,l^{-1}$ initial ΣPAH_{50}—explained in Methods section 'Initial oil exposure and acute mortality')[2,29]. This oil contains a relatively high proportion of three-ring PAHs (see Supplementary Fig. 1 and Supplementary Table 1 for details) typical for petrogenic pollution and believed to drive toxicity in marine biota[2,4,8,10,25]. We found that acute (24 h) exposure to crude oil HEWAFs during the PLP directly impairs the first four critical life-history stages, and has the potential to severely limit the successful recruitment and replenishment of larval fishes.

Results and discussion

During the PLP, fish travel with ocean currents across distances that may exceed hundreds of kilometres and are subject to exposure to numerous external stressors[22]. We exposed six species of pre-settlement reef fishes, spanning two evolutionarily distinct families (Pomacentridae and Lethrinidae) to crude oil HEWAFs and found significant reductions in survival within 24 h (two-way analysis of variance: $F_{2,70} = 21.42$, $P < 0.01$; Fig. 2). Across all species, 24 h exposure to 5.7 $\mu g\,l^{-1}$ ΣPAH_{50} reduced survival by 19.5 ± 4.5% relative to controls (mean ± s.e; Tukey's honest significant difference test: $P < 0.01$; Fig. 2), the magnitude of which was greatest for *Chromis atripectoralis*, *Pomacentrus bankanensis* and *Pomacentrus chrysurus* relative to *Pomacentrus amboinensis*, *Pomacentrus moluccensis* and *Lethrinidae* species (Supplementary Fig. 2). These results are consistent with previous studies on marine fish early life stages, which suggest that oil exposure causes elevated mortality in the low ppb ΣPAH_{50} range[10,25].

At the end of the PLP, larval reef fish approach the reef at night under the relative protection of darkness[22]. During the early hours of the following morning, individuals must rapidly find habitats that can provide food and shelter from predators[22,23]. This habitat selection is arguably one of the most critical determinants of subsequent survival, as individuals without adequate protection are rapidly preyed on[22,23]. To assess the impact of exposure to crude oil HEWAFs on larval settlement, we released groups of five naïve pre-settlement fish into a mesocosm simulating natural conditions, in which they had a choice to settle overnight on one of four habitat types. A total of 68 groups of *P. amboinensis* ($n = 100$) and *P. moluccensis* ($n = 240$) were used for this study and each was exposed to either 0, 2.5 or 5.7 $\mu g\,l^{-1}$ ΣPAH_{50} for 24 h. Habitat choices included high, medium and low complexity reefs (types 1, 2 and 3, respectively) and a plain sandy bottom (type 4) that provided no camouflage or refuge from predators. We then monitored settlement behaviour recurrently 10 min before and 10, 30, 60, 120 and 240 min after sunrise. While *P. amboinensis* and *P. moluccensis* demonstrated the greatest resilience to PAH_{50} exposure during the PLP, their settlement patterns were severely altered (*P. amboinensis*: $F_{3,79} = 4.22$, $P < 0.01$; *P. moluccensis*: $F_{6,191} = 3.88$, $P < 0.01$). Under control conditions, *P. amboinensis* and *P. moluccensis* strongly preferred the highly complex type 1 habitat, settling on this 73.5 ± 13.0% and 93.0 ± 4.4% of the time, respectively (mean ± s.e.m.). The less complex habitat types, 2 and 3, were only selected by the control fish 7.0 ± 4.4 and 14.0 ± 10.3% of the time, respectively. The type 4 habitat was completely avoided by both species (Fig. 3). Acute oil HEWAF exposure upturned these patterns, causing individuals to settle on the low complexity habitats that were previously avoided.

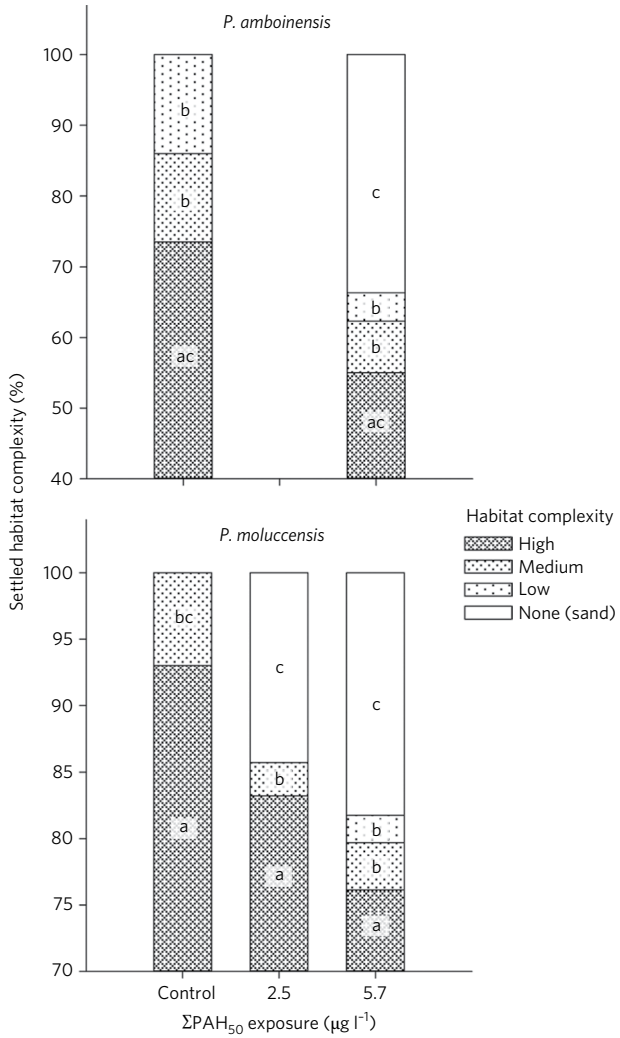

Figure 3 | Habitat settlement choice of crude oil HEWAF-exposed larval coral reef fish. Oil exposures are expressed as ΣPAH_{50} concentrations. Habitat choices included a high-, medium- and low-complexity reef and a plain sandy bottom, which provided no camouflage or refuge from predators. Significant differences between settlement choices are marked by letters.

After exposure to 5.7 μg l^{-1} ΣPAH_{50}, *P. amboinensis* was preferentially found in the open type 4 sand area in 33.7 ± 9.3% of cases, with declining settlement frequency on other habitat types (Fig. 3). Similarly, *P. moluccensis* selected the open type 4 sandy areas 18.2 ± 6.7% of the time, while a lower 2.5 μg l^{-1} ΣPAH concentration caused 14.3 ± 4.9% of *P. moluccensis* to settle on the sand (Fig. 3). Habitat selection in reef fishes is driven by a series of complex neurosensory and cognitive decisions, and these suboptimal settlement patterns highlight clear behavioural changes that may be related to impaired brain function.

Predation is a primary cause of mortality in larval reef fishes during settlement[23], and an instinctual ability to detect, avoid and escape predators is critical for survival[23]. Typical antipredatory behaviours in larval to adult reef fishes include shoaling to benefit from safety in numbers[30], avoiding unfamiliar open areas (for instance, via thigmotaxis[31]) and minimizing movements away from shelter once an appropriate habitat has been found[32]. Crude oil HEWAF exposure strongly affected all three of these antipredator behaviours in larval reef fishes. The 5.7 μg l^{-1} ΣPAH_{50} concentration reduced the shoal size of *P. amboinensis* by 27.6% ($F_{1,160} = 112.86$, $P < 0.01$, post hoc planned comparison (plc) $t = 2.36$, $P = 0.02$) and the shoal of *P. moluccensis* by 34.6% ($F_{2,330} = 7.01$, $P < 0.01$; plc $t = 2.33$, $P = 0.02$; Fig. 4). In *P. amboinensis*, this PAH concentration also increased the frequency of movement between habitats 2.0-fold (plc $t = 3.81$, $P < 0.01$; Fig. 4). In *P. moluccensis*, 5.7 and 2.5 μg l^{-1} ΣPAH_{50} concentrations caused a 2.4- and 2.2-fold increase in movements between habitats, respectively (plc $t_{5.7\,\mu g l^{-1}} = 3.10$, $P < 0.01$; $t_{2.5\,\mu g l^{-1}} = 2.89$, $P < 0.01$; Fig. 4), and exposure to 5.7 μg l^{-1} ΣPAH_{50} caused an overall 1.25-fold increase in the time individuals spent in open areas away from shelter ($F_{2,208} = 4.65$, $P = 0.01$), with the effect of PAH exposure varying among species ($F_{1,208} = 31.11$, $P < 0.01$; Fig. 4). Given the reduced number of individuals watching for predators and the increased exposure, these oil-driven behavioural changes are all likely to increase the rate of predatory detection and attack.

Once under attack from a predator, a larval fish must escape to survive. Fast escapes are routinely needed for the entire life cycle of a fish, and diminished performance severely reduces long-term survival[33]. A successful escape is widely thought to depend on response latency and the swimming speed and distances achieved during the first two axial bends of the tail (defined, respectively, as stage 1 and stage 2 of the escape response[33]). We used a mechanical stimulus and high-speed video to quantify these first two stages of the escape response. Our results showed no effect of oil HEWAF exposure on response latency relative to the control value of 15.9 ± 1.4 ms (*P. amboinensis*: $F_{2,112} = 0.51$, $P = 0.60$, $n = 117$; *P. moluccensis*: $F_{2,92} = 0.65$, $P = 0.52$, $n = 94$). Latency is primarily controlled by mechanically stimulated command neurons[33], suggesting that PAH compounds do not directly impair existing neuromotor connections despite altering gene expression related to neural development[27]. However, oil HEWAF exposure altered the escape behaviour by increasing the mean escape swimming speed and distance travelled in both species examined. At 5.7 μg l^{-1} ΣPAH_{50}, the mean escape swimming speed of *P. amboinensis* increased to 0.70 m s^{-1} relative to 0.63 m s^{-1} under control conditions ($F_{2,112} = 9.42$, $P < 0.01$), equating to a 13% increase in the total escape distance ($F_{2,112} = 10.53$, $P < 0.01$). For *P. moluccensis*, 5.7 and 2.5 μg l^{-1} PAH_{50} concentrations both increased the mean escape swimming speed, which reached 0.69 m s^{-1} relative to 0.57 m s^{-1} for the controls ($F_{2,92} = 17.70$, $P < 0.01$). This also increased the total escape distance by 21% ($F_{2,92} = 17.70$, $P < 0.01$).

We assessed the predation risk as a consequence of these altered settlement and antipredatory behaviours by releasing two individuals of *Pseudochromis fuscus*, a natural reef fish predator, into each mesocosm 1 h after the settlement trial concluded. We recorded larval fish survival after 10, 30, 60, 120 and 240 min and found a strong effect of oil HEWAF exposure on predation mortality ($F_{2,67} = 5.50$, $P < 0.01$). The response was the same for both species examined ($F_{2,67} = 0.51$, $P = 0.48$; Supplementary Fig. 3). Control fish had a 10.2 ± 2.8% mortality rate after 240 min, which was similar to natural conditions (Fig. 2; ref. [23]). However, fish exposed to 5.7 μg l^{-1} ΣPAH_{50} exhibited a 2.7-fold increase in mortality (Tukey's honest significant difference test: $z = 2.75$, $P = 0.01$; Fig. 2). These elevated mortality rates highlight the devastating outcome of adopting inappropriate or disproportionate behaviours during settlement.

After settlement, it is critically important that individuals undergo rapid growth, because smaller individuals are more prone to predation and less likely to survive to adulthood[24]. To assess the ecological effect of PAH exposure during the PLP on growth and latent mortality, we placed larval fish in a mesocosm to simulate natural coral shelters without predators, and then provided ad libitum access to food (*Artemia* species nauplii and zooplankton). Each mesocosm contained individuals exposed to one PAH_{50} concentration and one species (groups: *P. amboinensis*, $n = 29$; *P. moluccensis*, $n = 30$; and *P. chrysurus*, $n = 30$). Seven days post-settlement, fish exposed to 5.7 and 2.5 μg l^{-1} ΣPAH_{50} concentrations had grown 11.4 ± 1.6% and 10.2 ± 2.5% less ($F_{2,88} = 5.64$, $P < 0.01$) and were comparatively

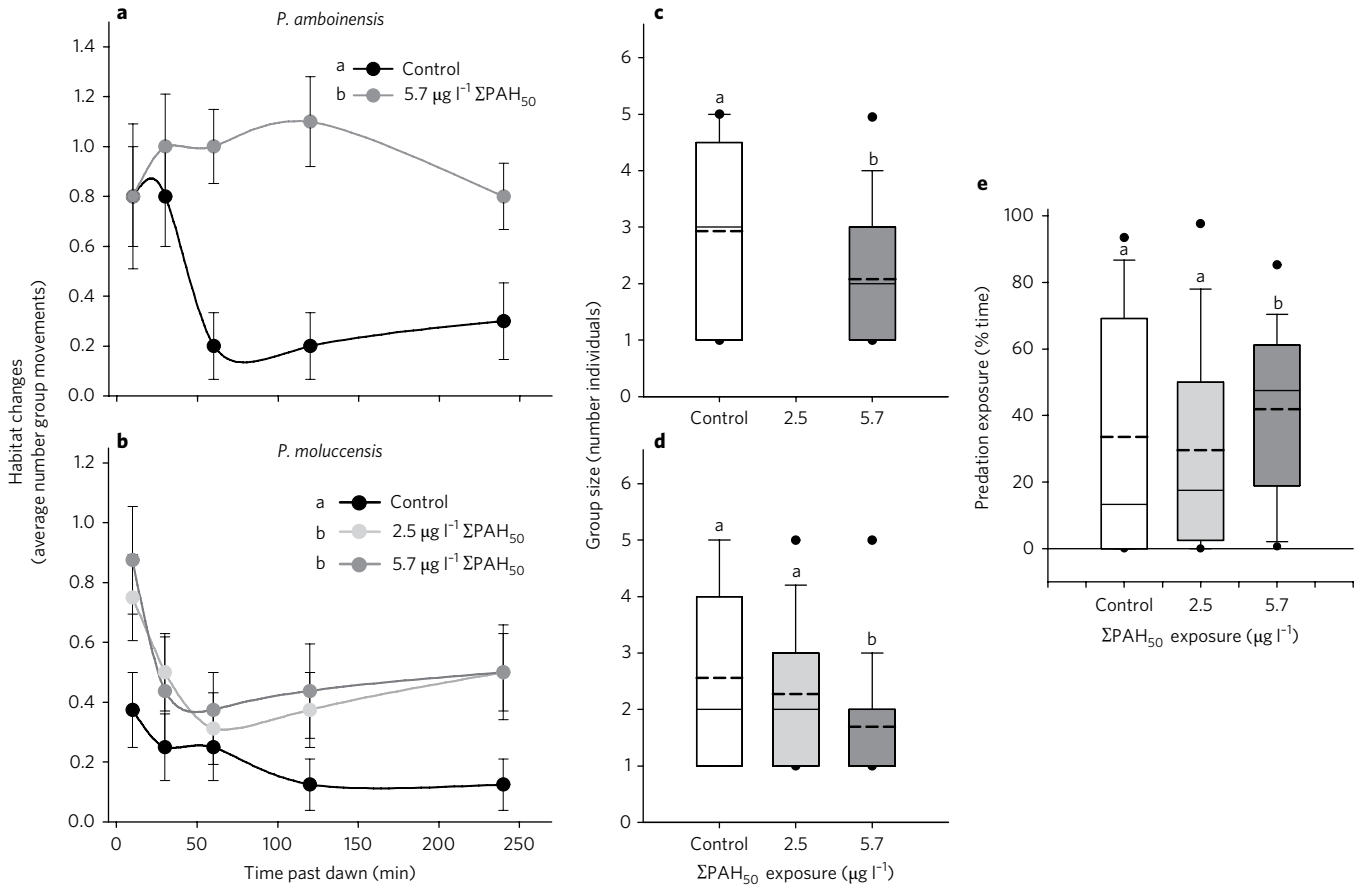

Figure 4 | Antipredatory behaviour of crude oil HEWAF-exposed larval coral reef fishes during settlement. Oil exposures are expressed as ΣPAH_{50} concentrations. **a**,**b**, Number of movements between habitats over time for *P. amboinensis*. (**a**) and *P. moluccensis* (**b**). Error bars, s.e.m. **c**,**d**, Changes in group size (shoaling) in larval *P. amboinensis* (**c**) and larval *P. moluccensis* (**d**) fishes exposed to different oil HEWAF concentrations. **e**, Changes in thigmotaxis. Significant differences are marked by letters. Box plots are structured in the same way as in Fig. 2.

smaller than control fish (post hoc Tukey's honest significant difference test: $P=0.02$ and $P<0.01$), irrespective of species ($F_{2,88}=2.52$, $P=0.09$; Supplementary Fig. 4). Exposure to 5.7 µg l^{-1} ΣPAH_{50} also caused an overall 11.8±3.3% increase in latent mortality, despite unlimited food and the absence of predators ($F_{2,88}=3.59$, $P=0.03$; Fig. 2), the magnitude of which differed by species ($F_{2,88}=27.7$, $P<0.01$; *P. amboinensis*: 17.5±6.1%; *P. moluccensis*: 11.4±5.7%; *P. chrysurus*: 6.4±5.3%; Supplementary Fig. 3). These results suggest that oil exposure during the early life stages of reef fishes has serious long-term consequences for their ecology and survival that are unrelated to habitat choice, predation or food availability. The behavioural changes observed also cannot explain the reduced growth and survival under ideal conditions with ample food, shelter and no predation, as the PAH-exposed fish were observed foraging at similar rates as the control fish. However, sublethal PAH exposure is known to cause heart deformities and reduced cardiac function in other marine fishes[10,25,26]. Although physiological injury was not quantified in this study, our results suggest serious latent physiological impacts leading to stunted growth and premature death.

Persistent organic pollutants, such as petrogenic PAHs, have been suggested as possible contributors to the rapid decline in health and resilience of many marine species and ecosystems worldwide[2]. The current paradigm of petrogenic PAH toxicity in fish suggests that cardiovascular injury is the root cause of subsequent ecological damage[10,25,26]. However, the behavioural changes that we recorded in crude oil HEWAF-exposed reef fish larvae were all related to higher-order brain functions, which provides strong evidence to suggest that chemicals found within oil directly impair cognitive processing in fish. While the impact of oil or waterborne PAHs on higher-order brain function has not previously been documented, our findings are supported by the existing literature. Oil exposure is known to alter gene expression relating to neurodegeneration and function in early life stages of fish[27]. Our results suggest that that PAH concentrations already found in many industrialized sections of tropical coral reefs worldwide, including parts of the Great Barrier Reef[14], Red Sea[12], Asia[2,11] and the Caribbean[13], are capable of causing physiological and cognitive impairment in early life stages of coral reef fishes. Critically, these impairments cause individuals to make inappropriate choices that severely alter the outcome of all early life-history events by increasing pre- and post-settlement mortality, reducing settlement success onto suitable habitat, increasing predator-induced mortality and reducing growth rates of exposed larval fish. These stages form the basis for all recruitment and maintenance of species diversity and abundance in coral reef ecosystems (for example, ref. [23]), and could have detrimental consequences for ecosystem health and resilience at large, particularly in areas subjected to industrial activities. Importantly, cognitive impairment associated with oil exposure has not previously been shown in marine biota. As such, our results highlight a suite of ecologically relevant endpoints, which—if affected at similarly low concentrations in other species and climate zones (such as temperate or polar)—are critical for a proper evaluation of the risks associated with increasing industrial activities within threatened ecosystems.

Methods

Species and collections. Before settling on a coral reef habitat, most reef fish have a PLP that typically lasts 16–28 days[28], but may be several months in some species or individuals far from other reefs. For this study, we used light traps to collect pre-settlement larval fish specimens as they came in to settle on the reef at night. Eight light traps were moored more than 30 m from a reef edge in 10–16 m of water off the lagoon reefs of Lizard Island in the northern Great Barrier Reef, Australia (14° 40′ S, 145° 28′ E). The light traps were left overnight and emptied each morning for juvenile fishes until all required pre-settlement specimens had been collected (Great Barrier Reef Marine Park Authority (GBRMPA) collection permit G12/35117.1). Targeted specimens were held in flow-through aquaria without substratum or shelters, which kept the larval fish in a pre-settlement stage in the water column for an additional 1–19 days until experimental protocols began. Non-targeted species were released back into the water at the collection site the following evening. A total of six pre-settlement species were selected for the experiments: *C. atripectoralis*, *P. amboinensis*, *P. bankanensis*, *P. chrysurus*, *P. moluccensis* and one *Lethrinus* species. These pre-settlement larval fish were chosen for their abundance in the light traps and tendency to inhabit a broad range of ecological niches on coral reefs. Due to differences in abundance, only *P. amboinensis* and *P. moluccensis* were used for all the experiments. Adult predatory fish (*P. fuscus*) were collected by scuba divers who carefully herded selected individuals into a barrier net and scooped them up in hand nets (Department of Primary Fisheries permit #170251 and GBRMPA collection permit G13/35909.1). This species naturally and ferociously predates on juvenile and newly settled reef fish. Individuals that hid within the corals were gently anaesthetized using a clove oil spray and collected with hand nets, which allowed for the targeted collection of individual fishes with minimal impact on adjacent reef organisms. Throughout the duration of the project, all fish species were maintained under James Cook University Animal Ethics Committee regulations (permit #A2255) according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the Queensland Animal Care and Protection Act 2001.

A total of 20 live coral and 20 dead rubble samples were also collected as natural substrate for the fish to settle on during the experimental protocols. The coral colonies consisted of branching staghorn *Acropora* coral (GBRMPA collection permit G15/38232.1), as these are highly abundant on the northern Great Barrier Reef and are typically used by many different species of larval fish as habitat. Each collected sample was less than 10 cm in diameter and only coral colonies and dead rubble of the appropriate size were taken, ensuring no large colonies were broken. To achieve this, each coral colony was gently dislodged from the substratum using a small chisel, whereas the collection of coral rubble was restricted to pieces already lying loose on the substratum of back reefs that typically contained large quantities of rubble substrate. All collected coral and rubble samples were held in flow-through aquaria under natural light conditions at the Lizard Island Research Station until the experimental protocols commenced (within two days). No coral or rubble samples were exposed to oil or other chemicals, and each sample was returned to its place of origin on the reef after completion of the experimental protocols (using GPS and reef topography). For all the holding facilities at Lizard Island, flow-through seawater was supplied directly from the adjacent reefs, and flow rates were used to maintain temperatures averaging 30.6 °C (austral summer averages for February and March) throughout the experimental period (range: 28.8–32.3 °C).

Initial oil exposure and acute mortality. Following standard international practices for toxicological research, exposure of the fish to oil was conducted using HEWAFs. HEWAFs were generated using a naturally weathered slick oil that was collected on 29 June 2010 from the hold of barge CT02404 in relation to the Deepwater Horizon Oil Spill in the United States in 2010 (Mississippi Canyon 252 Weathered Crude Oil, MSDS#03277). This oil was delivered to the University of Texas Marine Science Institute under the proper chain of custody and stored at 4 °C. Weathered oil was chosen for these studies owing to its environmental relevance. This is a heavy class C/D crude oil typical of oil spill scenarios involving oil exploration and large shipping vessels (see the United States Environmental Protection Agency oil descriptions at http://www2.epa.gov/emergency-response/types-crude-oil and the Material Safety Data Sheet for Mississippi Canyon 252 at http://gulfresearchinitiative.org/wp-content/uploads/2012/05/Weathered-Crude-Oil-MC252.062810.pdf). HEWAFs were generated at ambient air temperature (29.9 ± 0.2 °C) by mechanically blending 1 g of oil and 1 l of seawater using a hand blender for 30 s (Homemaker HB1913-C at rotation setting 5). The mixture was allowed to settle for 1 h in a separation funnel, after which the lower 85% was removed for use[26]. The fish were then placed in a circular 13.5 cm × 7 cm glass exposure aquarium containing 750 ml of 0, 5 or 10% HEWAFs in clean seawater with light aeration. Each exposure aquarium contained a density of pre-settlement larval fish of less than 0.3 g wet weight per 100 ml, which was equivalent to 5–25 individuals depending on the species and size of the individuals. The exposure aquarium was submerged in a flow-through bath at 30.6 ± 0.2 °C for thermal stability (mean ± s.e.m.; range: 29.2–32.1 °C) and all oil HEWAF exposures lasted 24 h. Due to the remote location of the Lizard Island Research Station, water samples could not be processed for ΣPAH directly from the exposure tanks. To properly anchor nominal HEWAF concentrations to ΣPAH concentrations, the exposure set up was replicated at the University of Texas Marine Science Institute. HEWAF samples were collected at 0 and 24 h and delivered on ice for commercial analysis within one week of collection (ALS Environmental protocol 8270D PAH_SIM). These analyses verified that the HEWAF preparations contained initial concentrations of 0, 2.5 and 5.7 $\mu g\,l^{-1}$ ΣPAH_{50} for the 50 PAH compounds most relevant for aquatic toxicity studies[2,10,25], which was equivalent to a geometric mean exposure of 1.0 and 2.1 $\mu g\,l^{-1}$ ΣPAH_{50} over 24 h (see the PAH compound list and details in Supplementary Table 1). After the 24 h exposure period (*C. atripectoralis*, $n = 90$; *P. amboinensis*, $n = 226$; *P. bankanensis*, $n = 180$; *P. chrysurus*, $n = 131$; *P. moluccensis*, $n = 323$; *Lethrinidae* species, $n = 45$), we recorded the mortality and transferred the remaining fish to flow-through holding tanks filled with clean, aerated flow-through seawater (that is, with no oil) until the experimental trials began (within 5–15 h). The holding tanks had no substratum structure for settlement, which kept the larval fish in a pre-settlement stage in the water column. All oil mixtures were disposed of according to institutional protocols.

Settlement behaviour. A set of mesocosm experiments were conducted on pre-settlement *P. amboinensis* and *P. moluccensis* to determine whether PAH exposure affected the capacity of reef fish to successfully find and settle on suitable habitat structures. A total of three patch-reef habitats were built inside each of four large, round 380 l mesocosm tanks (diameter: 110 cm, height: 50 cm) continuously supplied with flow-through seawater (mean temperature: 30.6 °C, range: 28.8–32.3 °C). Each patch reef was 10 cm in diameter and height, and constructed as either: type 1, a high-complexity reef consisting of 75–100% live coral structures, and providing good camouflage and more than eight places for the larval fish to hide; type 2, a medium-complexity reef consisting of dead rubble structures with 15–30% live coral, which provided some camouflage from predators and approximately four to six places to hide; or type 3, a low-complexity reef consisting of 100% dead rubble with poor camouflage from predators and only one or two identifiable places where larval fish could hide. Each mesocosm tank contained a sandy bottom with one high-, one medium- and one low-complexity patch reef, each placed 10 cm from the wall and equidistant from one another. The sand between the reefs was classified as habitat type 4, that is, no structural complexity, no camouflage from predators and no refugia in which to hide.

As juvenile reef fishes typically settle at night[23], for each trial, a total of five pre-settlement individuals of one single species were released into each mesocosm tank after sunset (around 21:00) and left to settle onto the patch reefs overnight. All larvae were released using a hand net in the centre of the mesocosm at equal distance from all three patch reefs. Settlement choice was then monitored recurrently starting at dawn (10 min before sunrise) and then 10, 20, 30, 60, 120 and 240 min after sunrise. Settlement choice was defined as the instantaneous location of each of the trial fishes on habitat types 1–4 at the time of monitoring. For each monitoring, we also recorded total group size on each habitat type and calculated the number of changes to habitat usage that had occurred since the last recording to ensure that settlement choice was not due to inactivity of the individuals (for instance, no selection). One-hundred *P. amboinensis* individuals were examined for settlement behaviour after 0 or 5.7 $\mu g\,l^{-1}$ ΣPAH_{50} exposure, whereas 240 *P. moluccensis* individuals were examined after 0, 2.5 or 5.7 $\mu g\,l^{-1}$ ΣPAH_{50} exposure, due to greater numbers of *P. moluccensis*.

Predation mortality. Predation is considered to be one of the main causes of mortality in juvenile reef fishes during the first 24–48 h of settlement[23]. Following each behavioural settlement experiment for *P. amboinensis* and *P. moluccensis* (see above), each mesocosm tank contained newly habitat-settled juvenile fishes. At 5 h past sunrise, two individuals of *P. fuscus* (total length: mean ± s.e.m., 7.6 ± 0.1 cm; range, 6.4–8.5 cm), which had been starved for 24 h, were released into each mesocosm tank and allowed to forage for prey. The habitat usage of both predators and prey, as well as the survival rates of the juveniles, were then monitored recurrently after 10, 30, 60, 120 and 240 min. Individuals that could no longer be observed within the mesocosm tanks were deemed to have been preyed on. At the end of the 240 min trial, all predators and the remaining juvenile fishes were removed from the mesocosm tanks, taking care to thoroughly dismantle and examine all patch reefs for hiding individuals. Predation rates were then calculated as the number of prey observed at the beginning of the predation trial, minus the number of prey remaining at the end of the predation trial. The sand within the mesocosm tanks was then mixed and the tank sides scrubbed to remove algal growth, whereafter the patch reefs were reconstructed. Each mesocosm tank was then thoroughly flushed for at least 5 h at a rate of approximately 10 l min^{-1}. A total of 24 predators were used for this experiment, which allowed individuals to be randomized between tanks for each experiment. Corals within the patch reefs were also replaced every 5–7 days to ensure the predators remained unfamiliar with the specific hiding places on each patch reef.

Thigmotaxis and fast start. Thigmotaxis (for instance, 'wall hugging' as an indication of shyness in a novel environment[31]) and fast-start escape performance (necessary to evade predator attacks[33]) were examined for 117 *P. amboinensis* and 94 *P. moluccensis* individuals after 0, 2.5 and 5.7 $\mu g\,l^{-1}$ ΣPAH_{50} exposure. Both performance indices were examined in a transparent circular acrylic arena

(diameter: 200 mm; height: 70 mm) within a large opaque-sided plastic tank (585 mm × 420 mm × 330 mm; 60 L) with a transparent Perspex bottom to allow responses to be filmed from below using the silhouettes of the fish. The water level was maintained at 50 mm to reduce movements in the vertical plane, and the water in the arena was emptied and refilled with flow-through seawater after every fourth trial to maintain water quality and temperature. For the fast-start experiment, the juvenile fishes were startled by a mechanical stimulus that was suspended within a 4.0 cm white polyvinyl chloride pipe placed directly over the centre of the arena. The stimulus was released into the water using an electromagnet and remained invisible to the juvenile fish until the falling stimulus touched the water surface. The stimulus consisted of a 100 g black bullet-shaped steel cylinder with a diameter of 20 mm and a length of 45 mm, and a lanyard prevented the stimulus from hitting the bottom of the arena, thus ensuring the escape stimulus was based on the mechanical stimulus breaching the water surface.

At the beginning of the trial, a pre-settlement fish was introduced to the arena and allowed to familiarize itself with the surroundings for 5 min. At the end of the 5 min period, routine activity (used to determine thigmotaxis) was recorded as a silhouette from below, at 30 frames per second and 680 × 480 resolution for 2 min. After the 2 min period, high-speed video recordings for fast-start estimations were started at 480 frames per second and 226 × 160 resolution. Both video sequences were recorded using a Casio ZR1000 camera mounted to a Casio Exilim HS 24 mm wide optical lens with a zoom of 78 mm, and stored to a SanDisk Ultra 32 gb class 10 SD card. The mechanical stimulus was only released once the juvenile fish swam within two body lengths of the central stimulus polyvinyl chloride pipe (a distance of 18–20 mm). This allowed all individuals to move an equal distance in any direction and standardized the fish position relative to the stimulus. No differences were detected between treatments in the distance of the fish relative to the startle stimulus (*P. amboinensis*: $F_{1,112} = 0.22$, $P = 0.80$; *P. moluccensis*: $F_{2,90} = 0.22$, $P = 0.79$). To ensure a standardized protocol, prey escape variables were only measured when prey performed a C start (commencement of a fast start that results in the individual forming a C shape).

Thigmotaxis of individuals in a novel environment was analysed using the 2 min, 30 frames per second video sequences[31]. By marking the location of an individual every second, all test subjects were assessed for the proportion of time spent swimming along the wall of the arena (that is, within 2.5 body lengths of the wall) or further into the centre of the arena. Kinematic variables associated with the fast-start response were analysed using the image analysis software ImageJ (v. 1.6; https://imagej.nih.gov) with a manual tracking plugin. The point at which each fish was tracked was standardized by following the same point on each fish (that is, the position directly behind the eyes that corresponds to the thickest part of the body). We chose to standardize the tracking using this point of the body as it was the most stable and easiest to track owing to the small size of the larvae. The 480 frames per second videos were used to examine fast-start reaction latencies from the time of the stimulus hitting the water surface, as well as average escape swimming speeds and total distances covered during stages 1 and 2 of the fast-start escape (here defined as the first 24 ms). All video analyses were conducted blind (that is, the observer did not know the oil HEWAF exposure concentration of the individuals).

Growth and prolonged mortality. Growth potential and latent mortality following PAH exposure were examined by placing 90 groups of juvenile fishes in identical 10 l (22 cm × 22 cm × 22 cm) aquaria, each containing a 10 cm diameter × 10 cm high coral rubble shelter. Growth was tested for three species (*P. amboinensis*, $n = 129$; *P. chrysurus*, $n = 139$; and *P. moluccensis*, $n = 159$) at three exposure concentrations (0, 2.5 and 5.7 $\mu g\,l^{-1}$ ΣPAH_{50}) with 8–12 replicates for each treatment. The stocking density of the juvenile fishes was kept below 0.1 g wet weight per 100 ml, which was equivalent to an initial group size of 3–7 individuals per aquarium, depending on the species and size of the individuals. A total of 38 aquaria were used, enabling all trials to be initiated within a 16 day period. Each aquarium was supplied with flow-through seawater, which contained small planktonic food particles from the reef, and augmented twice daily with a surplus of newly hatched *Artemia* species nauplii. The collective group wet mass was measured just before and at the end of each seven-day grow-out period. Juvenile growth rates were calculated at the end of the period as an average gain in mass per individual. Mortality was tallied at the end of the growth period.

For all the experiments, exposure concentrations and species were randomly mixed to minimize confounding factors related to, for example, the holding duration or ambient water temperature on the day of trial. Due to the limited number of available specimens, some individuals were moved to the grow-out experiment after being tested for fast-start and settlement behaviour. These individuals were allowed to rest for 24 h between each experiment. After completion of the experiments, all remaining oil HEWAF-exposed fish were euthanized using an overdose of clove oil following standard ethical protocols for the humane treatment of animals (James Cook University Animal Ethics Committee regulations; permit #A2255).

Statistical analyses. The effect of PAH_{50} exposure on fish acute mortality, growth and latent mortality were analysed using two-way analyses of variance for each experiment, with species and PAH exposure concentration as fixed factors.

The effect of PAH exposure on routine thigmotaxis and kinematic variables during the fast-start escape was examined using one-way multivariate analyses of variance, incorporating species and PAH_{50} exposure as fixed factors. Settlement choice, settlement behaviour and predation mortality were examined using general linear mixed models (GLMM). GLMMs are highly robust to non-independence of data points obtained for the same individual and can produce unbiased estimates of variance and covariance[34]. In these models we treated species, habitat type and exposure concentration as fixed effects. Groups were nested within exposure concentrations and treated as random effects. To assess the validity of the mixed effects analyses, we performed likelihood ratio tests comparing the models with fixed effects with the null models with only the random effects. We rejected results in which the model, including fixed effects, did not differ significantly from the null model. The significance of main effects was estimated using Markov chain Monte Carlo *P* values[35]. This method is robust to the fact that the exact degrees of freedom cannot be calculated in complex GLMM designs[35]. Significant factors and interactions were examined using post hoc Tukey's honest significant difference test or planned comparisons, followed by false discovery rate corrections for type I error. Normality and homogeneity of variance were confirmed using Bartlett's test and visual inspections of plots of residuals against fitted values. We used log, sqrt, asin(sqrt) and boxcox transformations where appropriate. All data were analysed in SigmaPlot (v. 12; https://systatsoftware.com/products/sigmaplot/), Statistica (v. 12; www.statistica.io) and R (v. 3.2.2; https://www.r-project.org/) using the R packages lme4, languageR, LMERConvenienceFunctions, multcomp and lsmeans.

Data availability. Data are publicly available through the GMRI Information and Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (doi:10.7266/N7NK3C36 and doi:10.7266/N7T72FH4).

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Author contributions

J.L.J. and A.J.E. conceived the idea. J.L.J. designed the experiments. J.L.J., B.J.M.A. and J.L.R. performed the experiments. J.L.J. and B.J.M.A. analysed the data. J.L.J. wrote the manuscript with input from B.J.M.A., J.L.R. and A.J.E.

Competing interests

The authors declare no competing financial interests.

Additional information

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