Neuroactive pollution alters shoaling in fish, but group phenotype matters

Authors

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Abstract

Collective behavior governs how animal groups evade predators, forage, navigate, and share information. Yet it is rarely the focus of research on human-induced environmental change, particularly in the case of chemical pollution. Here, we show that exposure to the common neuroactive pollutant temazepam disrupts shoaling dynamics in fish (*Poecilia reticulata*), with effects shaped by group phenotype. Using 111 groups from artificial-selection lines divergent in collective coordination, we quantified group dynamics and the local interaction rules from which they emerge. In wild-type groups, temazepam weakened alignment and attraction between neighbours, reducing group polarisation, speed, and cohesion, particularly during early group establishment when social information is rapidly integrated. Conversely, groups selected for high coordination maintained or increased these traits. We provide the first evidence that neuroactive pollution alters local interaction rules in ways that scale up to collective motion, and that group phenotype can buffer or amplify pollutant effects.

INTRODUCTION

Collective behaviours arise from local interactions that produce emergent group-level patterns, such as the synchronised motion of fish schools (*1*), living raft formation of fire ants (*2*), and the coordinated, cannibalism-driven marching of locust swarms (*3*). For animals that are largely solitary, to those that form obligate aggregates, collective behaviours play an essential role at some stage of their life history, with profound ecological consequences (*4*, *5*). While these group-level behaviours are increasingly well described from mechanistic and evolutionary perspectives, their responses and robustness to rapid, human-induced environmental change remain unclear. This knowledge gap is particularly apparent in the context of chemical pollution (*6*), which represents one of the fastest-growing threats to global biodiversity (*7*, *8*).

A range of common chemical pollutants, including pharmaceuticals, pesticides, and industrial chemicals, are known to alter animal behaviour at the individual level, yet whether and how these effects scale up to influence collective behaviours—and the local interaction rules that govern them (alignment and attraction)—remain poorly understood (*9*). Equally unclear is whether all groups, regardless of their constituent behavioural phenotypes, respond to pollutants in the same way. Within species, collective behaviour can vary substantially among populations, with ecological pressures such as predation risk, habitat structure, and resource distribution shaping divergent collective phenotypes (*10*, *11*). These differences are often repeatable and heritable (*12*), yet this principle has rarely

been extended to collective behaviour, much less to the effects of chemical pollutants. Whether pre-existing differences in collective behaviour shape responses to contaminant exposure, therefore, remains unknown. Such variation matters because pollution is unlikely to affect all populations equally; instead, its ecological consequences may depend on the collective phenotypes they express. Identifying how collective phenotypes shape contaminant sensitivity could therefore help refine ecological risk assessments, moving beyond average species-level responses toward more precise predictions of which populations are most sensitive to behavioural disruption.

Here, we tested whether and how exposure to the psychoactive pharmaceutical temazepam, a benzodiazepine and central nervous system depressant common in aquatic environments (*13*), affects collective motion and the social interaction rules that underlie shoaling behaviour in a freshwater fish, the guppy (*Poecilia reticulata*). Specifically, we addressed three questions: (1) Does temazepam exposure alter collective behaviour at the group level? (2) Are group-level changes underpinned by shifts in the local social interaction rules amongst individuals? (3) Are any such pollutant effects mediated by the baseline shoaling characteristics of the group?

To address these questions, adult female guppies were exposed to one of three conditions for 10 d: unexposed treatment (i.e. solvent control), a low temazepam dose (0.11 µg L^{-1}; **Fig 1A**), or a high temazepam dose (1.07 µg L^{-1}; **Fig 1A**). The low dose represents the upper end of reported surface water concentrations (range: <0.001–1.38 µg L^{-1}; (*13*), while the high dose represents the upper end of reported effluent concentrations (range: <0.001–1.86 µg L^{-1}; (*13*)). The social behaviours of 111 groups, each with five unfamiliar individuals (N = 555), were then quantified using AI-assisted unmarked video tracking (*14*). To examine how baseline shoaling characteristics modulate the impacts of exposure, we used fish from artificial selection lines bred for elevated shoal alignment (hereafter, 'social-type' lines) over three generations (*15*) and compared their responses with control lines representing standing variation in the source population (hereafter, 'wild-type' lines). These selection lines, with expression divergences of 15% in shoal alignment (*15*), gave us a unique opportunity to directly explore the role of collective phenotypes in mediating responses to chemical exposure, while linking any emergent group-level changes to their underlying interaction rules.

We predicted that temazepam, as a gamma-aminobutyric acid $(GABA)_A$-modulating benzodiazepine, would dampen central nervous system arousal and reduce the gain of

sensory-to-motor integration that is key for accurate and rapid collective motion. Therefore, temazepam was expected to decrease responsiveness to local social cues, consistent with previously reported behavioural effects of other benzodiazepines in fish (*16*–*18*). Thus, regarding question 1, exposure was expected to weaken shoaling coordination, as reflected in reduced shoal alignment, swimming speed, cohesion, and mean shoal size, as well as destabilised leadership. For question 2, we further predicted that group-level changes in shoaling behaviour would be underpinned by changes at the individual level, with weakened local interaction rules, such as alignment and attraction interactions among neighbours, providing a potential mechanistic link for pollutant-induced disruption of collective behaviour (*15*). Lastly, for question 3, we predicted that these effects would be more pronounced in the social-type groups, which rely more heavily on fine-tuned and strongly coordinated shoaling behaviour (*15*, *19*), and may therefore be more susceptible to disruption, leading to a disproportionate breakdown in collective coordination.

RESULTS

Temazepam exposure alters collective motion and weakens social interactions

We quantified the effects of exposure on collective behaviour by tracking groups of five unfamiliar fish for 34 min following release into a novel testing arena (see Materials and Methods: Behavioural quantification and trajectory processing for details). From these trajectories, we extracted five group-level metrics describing complementary aspects of shoal organisation and coordination: polarisation (directional alignment), shoal speed (the magnitude of the mean velocity vector of shoal members), cohesion (mean nearest-neighbour distance), average shoal size (individual-weighted mean dynamic shoal size), and leadership turnover (the rate at which the foremost individual along the shoal's heading changed; see Materials and Methods: Behavioural quantification for details).

We focused on how these metrics change after a novel group of fish is introduced, when coordination is challenging and must be rapidly established. If exposed individuals are slower to coordinate following group introduction, even transient disruption could accumulate across repeated shoal reassembly events, as is common in species with fission–fusion social groups like *P. reticulata* (*20*).

Temazepam exposure disrupted multiple components of collective motion, with effects that were strongest during early shoal establishment. In wild-type fish, high-dose exposure reduced polarisation intermittently across the trial, including immediately after group

introduction, mid-trial, and toward the end of the observation period. The largest contrast occurred at group introduction (i.e. minute 0), when unexposed groups were 6.6% [89% CrI: 1.6%, 11.9%] more polarised than high-dose groups. In contrast, low-dose exposure produced no clear effect on polarisation (**Fig. 1B; Table S1**). Shoal speed showed a similar pattern, with high-dose groups moving more slowly than unexposed groups shortly after the groups were introduced. This contrast was strongest at group introduction, when unexposed groups were 40.2% [9.9%, 85.5%] faster than high-dose groups (**Fig. 1C; Table S1**). Low-dose exposure also transiently reduced cohesion during mid-trial (control–low peak $\Delta = -3.6\%$ [−6.4%, −0.3%] at minute 16.0), whereas average shoal size and leadership dynamics did not differ credibly across treatments (**Fig. 1E–F; Table S1**).

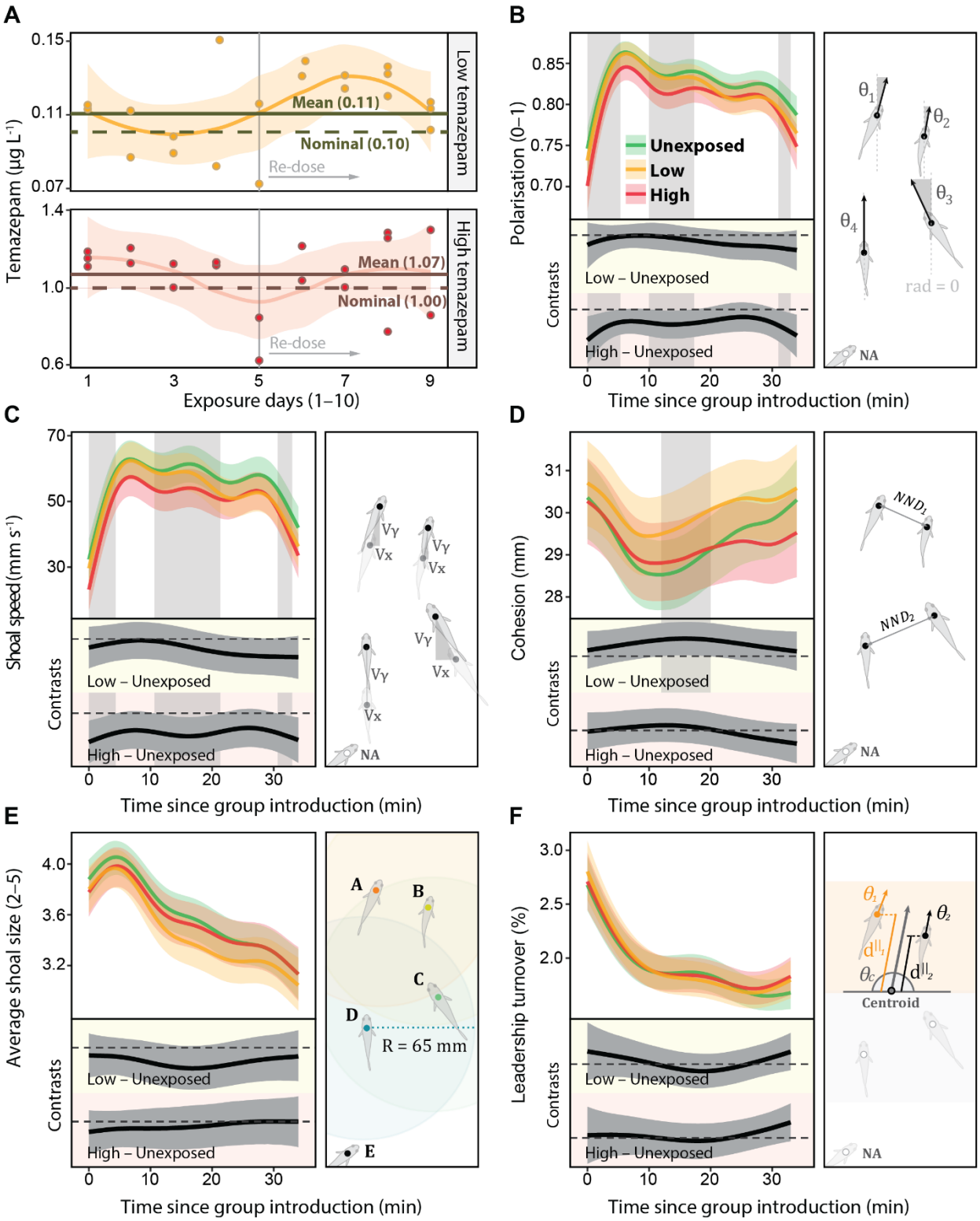

Fig. 1. **Temazepam exposure alters group formation and collective motion in wild-type fish. A**, Temporal profile of temazepam concentrations in exposure aquaria for the low (yellow; *n* = 20) and high (red; *n* = 20) treatments, estimated from grab samples taken

across multiple tanks (see Supplementary methods for details). Points show measured concentrations (μg L^{-1}) across exposure days (1–9). Solid curves indicate Locally Weighted Scatterplot Smoothing trends, with horizontal solid lines showing observed mean concentrations (low: 0.11 μg L^{-1}; high: 1.07 μg L^{-1}) and dashed lines indicating nominal targets (0.10 and 1.00 μg L^{-1}). The vertical grey line denotes a 20% re-dose on day five. **B–F**, Time-resolved dynamics of collective motion following group introduction. Bayesian hierarchical generalised additive model estimates of five group-level metrics: polarisation (i.e. directional alignment; **B**), shoal speed (**C**), cohesion (mean nearest-neighbour distance; **D**), average shoal size (**E**), and leadership turnover (F), plotted as a function of time since group introduction (min). Coloured lines represent marginal estimated medians (unexposed: green; low: yellow; high: red), with shaded ribbons indicating 89% credible intervals (CrIs). Grey vertical bands indicate periods where pairwise contrasts between treatments, within a selection line, are credibly different (89% CrIs exclude zero). Lower panels show posterior contrasts (low – unexposed; high – unexposed), with ribbons indicating 89% CrIs and dashed horizontal lines denoting zero difference.

To understand the behavioural mechanisms underlying these group-level changes, we quantified local interaction rules among nearest-neighbour dyads. Guppy movement is structured as a sequence of short, discrete bursts separated by passive glides, with directional changes occurring at the start of these bursts (*15*). Therefore, these burst events provide temporally discrete decision points, allowing us to link changes in movement direction to the position and orientation of a nearby individual (**Fig. 2A–B**). If shoaling is organised around an optimal inter-individual distance, alignment should be strongest near optimal spacing, whereas attraction should restore spacing when neighbours deviate from it (*21*). Quantifying how alignment and attraction vary with nearest-neighbour distance thus allows us to assess whether exposure alters both the distance range over which these interaction rules operate (i.e. spatial tuning) and the strength of the response.

Temazepam exposure modified how individuals responded to social cues. Alignment—turning to match the direction of a neighbour's heading—was weaker under high-dose exposure, particularly around the distance at which it is normally strongest (**Fig. 2D; Tables S2–3**). The high-dose peak alignment angle was 7.8% [3.3%, 12.3%] larger than the unexposed estimate, indicating weaker coupling, with credible differences spanning multiple neighbour-distance ranges. Attraction—turning toward the position of a neighbour—was also affected; low-dose exposure increased the peak coupling angle by 6.4% [0.6%, 12.6%] and produced credible differences over two neighbour-distance ranges (**Fig. 2E; Tables S2–3**). Importantly, exposure did not alter the frequency of burst events,

indicating that the observed changes in alignment and attraction were not explained by changes in burst-glide activity (**Fig. 2C; Tables S4–5**). Together, these results indicate that temazepam disrupts collective motion primarily by weakening alignment interactions between individuals under the high-exposure condition; attraction interactions were affected primarily in the low-dose treatment, potentially explaining the reduction in shoal cohesion seen only at the low dose, and burst frequency was unchanged. This degradation of local interaction rules likely underpins the observed reductions in group-level coordination seen in the early stages of group introduction.

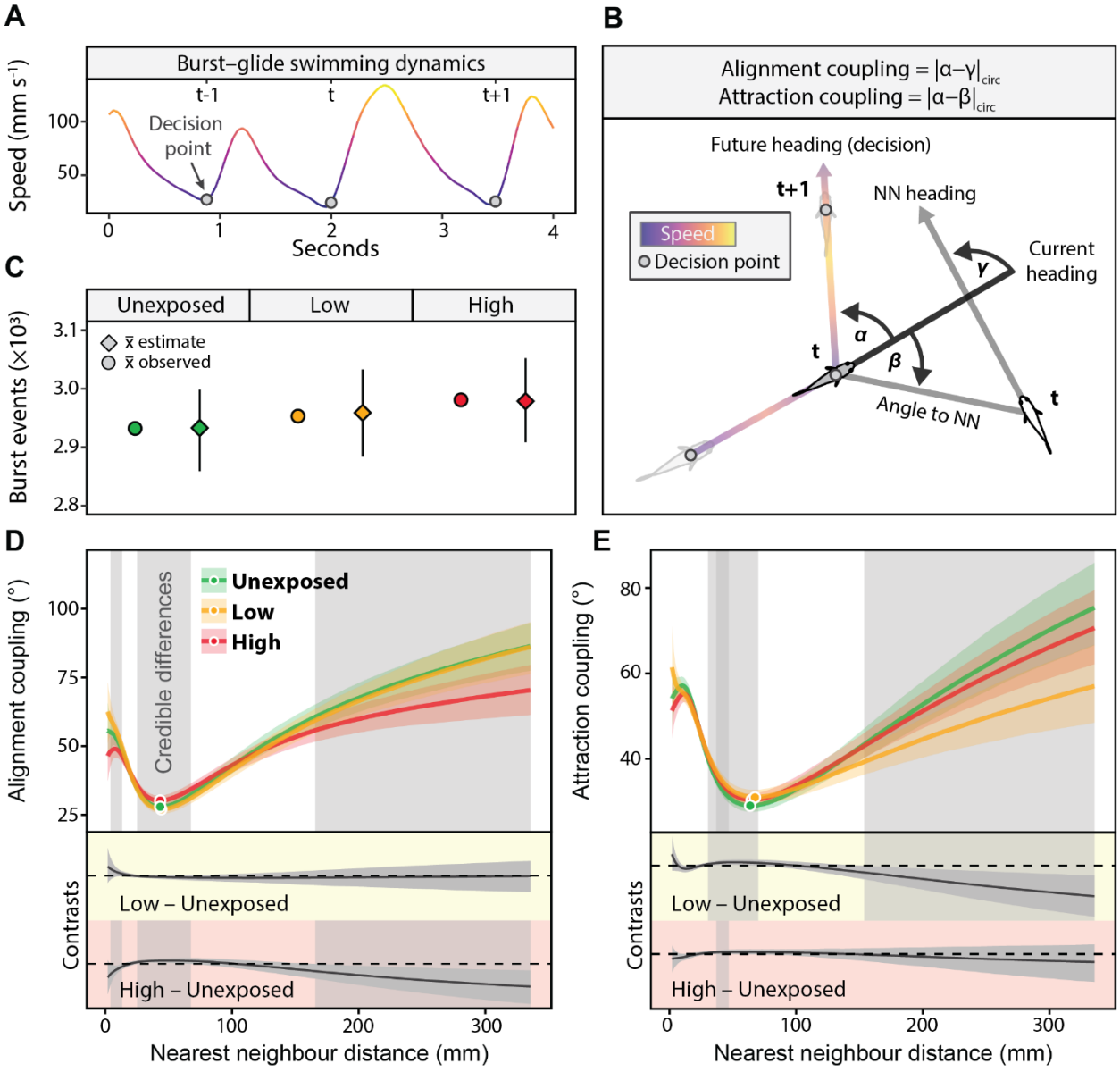

Fig. 2. Temazepam alters local alignment and attraction rules. A, Representative speed time series illustrating burst–glide swimming dynamics. Circles denote decision events,

and coloured trajectories indicate instantaneous speed (mm s^{-1}). **B**, Schematic of the local decision geometry underlying social interactions. At time t, the focal fish (grey) adjusts its heading based on information about the distance and direction of its nearest neighbour. The turning response (α) is defined as the change in heading from t to t+1. The angular position of the nearest neighbour (NN; white) relative to the focal heading defines the angle representing maximum attraction (β), while the difference in heading between focal and neighbour defines the maximum alignment angle (γ). Alignment and attraction coupling are quantified as the circular angular differences $|\alpha-\gamma|_{circ}$ and $|\alpha-\beta|_{circ}$, respectively. **C**, Total burst events across trials under unexposed, low (0.11 μg L^{-1}), and high (1.07 μg L^{-1}) temazepam exposure. Points indicate observed means (circles) and estimated marginal means (diamonds), with vertical bars representing 89% credible intervals (CrIs). **D**, **E**, Posterior marginal mean estimates of alignment coupling (**D**) and attraction coupling (**E**) as a function of nearest-neighbour distance under unexposed, low, and high exposure conditions. Lines show the posterior median estimates, with shaded ribbons indicating 89% CrIs, and coloured circles denote posterior medians of the draw-specific minima. Grey vertical bands denote neighbour-distance ranges where treatment contrasts are credibly different (89% CrIs exclude zero). Lower panels show posterior pairwise contrasts (low – unexposed; high – unexposed), with shaded ribbons indicating 89% CrIs and dashed horizontal lines denoting zero difference.

Exposure effects depend on group phenotypic composition

To test whether the effects of exposure were mediated by baseline shoaling characteristics, we compared the responses of wild-type groups with those of the social-type groups (i.e., those artificially selected for increased shoal alignment; for details, see Materials and Methods, Selection lines). Under unexposed conditions, differences between social and wild-type lines were consistent with the prior selection responses (*15*). Social-type groups exhibited higher polarisation and swimming speed during early group establishment. These differences were strongest early in the trial: at group introduction, social-type groups were 5.9% [1.5%, 10.7%] more polarised, and their peak speed difference was 19.4% [2.8%, 40.3%] at minute 1.7 (**Table S1**). These differences were most evident in the early stages of the trial and then diminished as groups converged over time (**Fig. 3B–C**). Although cohesion and shoal size did not differ detectably between line-types, these traits were not direct targets of selection, but rather additional shoaling parameters included here to assess correlated behavioural responses (**Fig. 3D–E**; **Table S1**).

Temazepam exposure drove a marked divergence between wild-type and social-type groups. While wild-type groups showed reductions in polarisation and speed under high

exposure and reduced cohesion under low exposure, social groups showed smaller or qualitatively different responses (**Fig. 3B–F**). The maximum difference in polarisation between the selection lines increased progressively with exposure, from 5.9% [1.5%, 10.7%] in unexposed groups to 8.3% [3.7%, 13.6%] under low exposure and 13.6% [8.0%, 19.6%] under high exposure (**Fig. 3B**; **Table S1**). Speed showed a non-monotonic pattern, with the difference between line-types being slightly smaller under low exposure (17.2% [4.7%, 32.6%]) than under unexposed conditions (19.4% [2.8%, 40.3%]), but increased markedly under high exposure (79.2% [35.0%, 152.5%]; **Fig. 3C**; **Table S3**). Shoal size did not differ detectably between lines under unexposed conditions (peak Δ = 5.6% [−1.5%, 13.2%]), but did diverge under both low (8.9% [3.2%, 14.8%]) and high exposure (10.2% [4.3%, 16.6%]; **Fig. 3D**; **Table S1**), indicating that exposure can reveal line-type differences in shoaling characteristics that are not evident under unexposed conditions. Cohesion remained similar between lines across all treatments (**Fig. 3E**; **Table S1**). Leadership turnover also showed time-dependent line differences. Under unexposed conditions, credible differences were restricted to the end of the trial (minutes 29–33). Under low exposure, social-type groups had 20.8% [9.8%, 29.7%] lower peak turnover than wild-type groups at group introduction, with differences during minutes 0–4.7 and 29–33. Under high exposure, the line difference emerged late (minutes 29.3–33), reaching 15.5% [2.6%, 25.8%] lower turnover in social-type groups at minute 33.0 (**Fig. 3F**; **Table S1**). These results suggest that a collective phenotype can modulate the behavioural effects of temazepam, leading to distinct pollutant responses across social and wild-type groups.

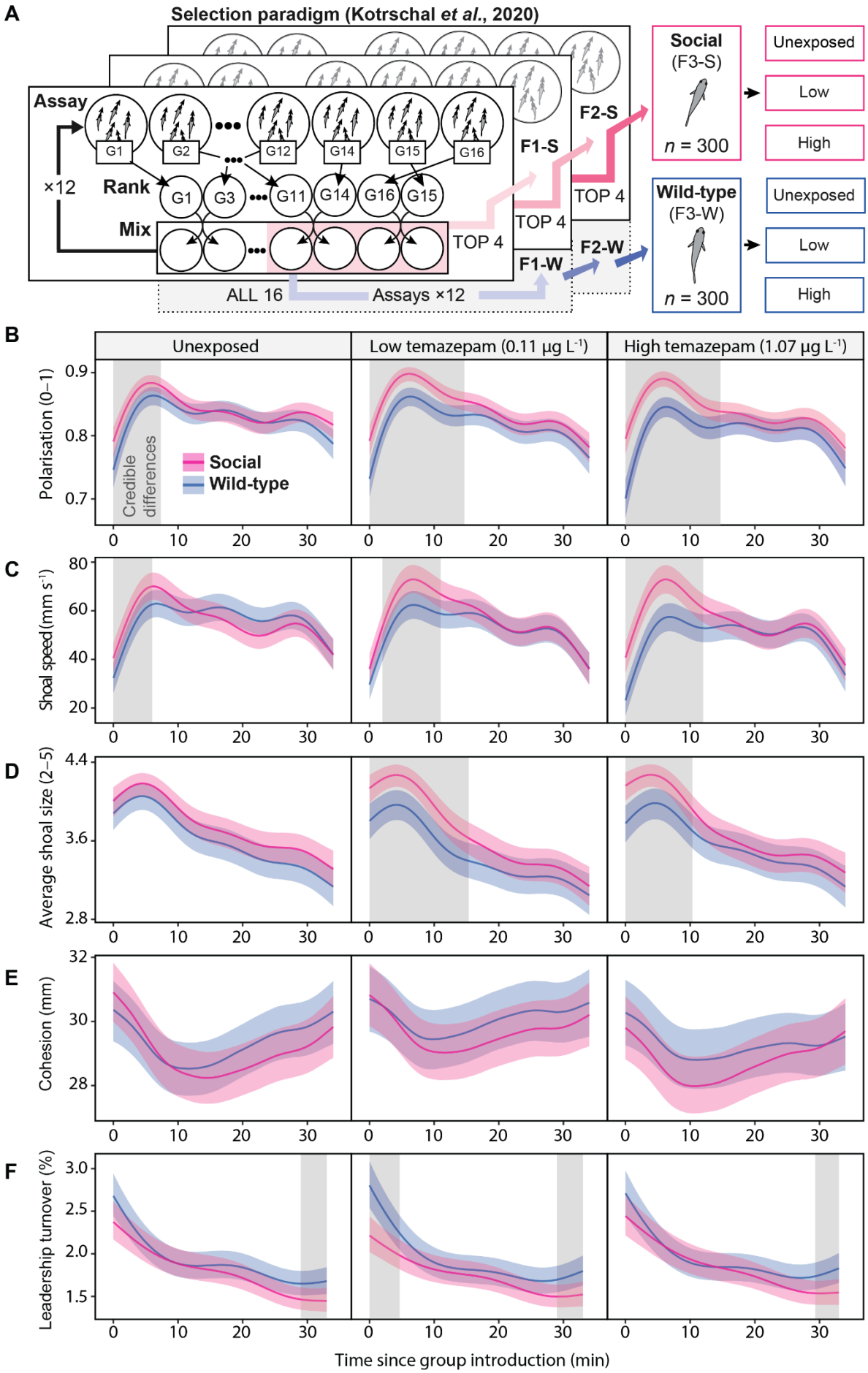
A
Selection paradigm (Kotrschal et al., 2020)
Assay
G1
G2
G12
G14
G15
G16
×12
Rank
G1
G3
G11
G14
G16
G15
Mix
TOP 4
F1-S
F2-S
TOP 4
TOP 4
F1-W
F2-W
ALL 16
Assays ×12
Social
(F3-S)
n = 300
Wild-type
(F3-W)
n = 300
Unexposed
Low
High
Unexposed
Low
High
B
Unexposed
Low temazepam (0.11 µg L-1)
High temazepam (1.07 µg L-1)
Polarisation (0–1)
Credible differences
Social
Wild-type
C
Shoal speed (mm s-1)
D
Average shoal size (2–5)
E
Cohesion (mm)
F
Leadership turnover (%)
Time since group introduction (min)

Fig. 3. **Exposure amplifies divergence in collective motion between selection lines. A**, Schematic of the artificial selection paradigm used to generate social and wild-type lines (*15*). In each generation, females were repeatedly sorted based on group polarisation across 16 groups (G1–G16). Following each assay, individuals were ranked, partially mixed with adjacent groups, and re-assayed over 12 rounds. After the final round, individuals from the top-ranked groups were used to found the social lines, while individuals from the remaining groups founded the wild-type lines. This procedure was repeated across three generations to generate independent replicate lines, from which experimental fish were derived. **B–F**, Time-resolved dynamics of collective motion following group introduction under unexposed, low (0.11 μg L^{-1}), and high (1.07 μg L^{-1}) concentrations of temazepam exposure. Within each treatment, groups were composed of either social (pink) or wild-type (blue) fish. Coloured lines show marginal estimated medians from Bayesian hierarchical generalised additive models, with shaded ribbons indicating 89% credible intervals (CrIs). Rows show: polarisation (i.e. directional alignment; **B**), shoal speed (**C**), average shoal size (**D**), cohesion (mean nearest-neighbour distance; **E**), and leadership turnover (i.e. change in leadership; F), plotted as a function of time since group introduction (min). Grey vertical bands indicate time periods where contrasts are credibly different (89% CrIs exclude zero).

These phenotype-dependent effects were also evident in the local interaction rules that generate collective motion. In wild-type fish, temazepam weakened alignment responses (**Fig. 2D**), indicating reduced precision in how individuals matched the heading of their neighbours. In social-type fish, high exposure strengthened peak alignment by 6.0% [0.5%, 11.7%], whereas peak attraction did not change credibly across treatments (**Fig. S1**; **Table S3**). Consequently, differences between wild-type and social-type groups widened under exposure, especially at the high dose. Under control conditions, social-type fish showed a 4.7% [0.1%, 9.8%] stronger peak alignment response, but did not show meaningfully stronger attraction than wild-type fish (a non-credible difference of 2.6% [−3.0%, 8.1%];**Fig. 4**; **Table S3**). Under high exposure, social-type fish showed an 18.6% [13.0%, 24.5%] stronger peak alignment response and a 10.3% [4.8%, 16.5%] stronger peak attraction response than wild-type fish (**Fig. 4**; **Table S3**). Together, these results show that the effects of temazepam on collective behaviour are not uniform but depend strongly on the baseline shoaling characteristics of a group. Rather than homogenising collective behaviour across groups, exposure amplified pre-existing differences, driving opposing responses in collective dynamics and the interaction rules that generate them.

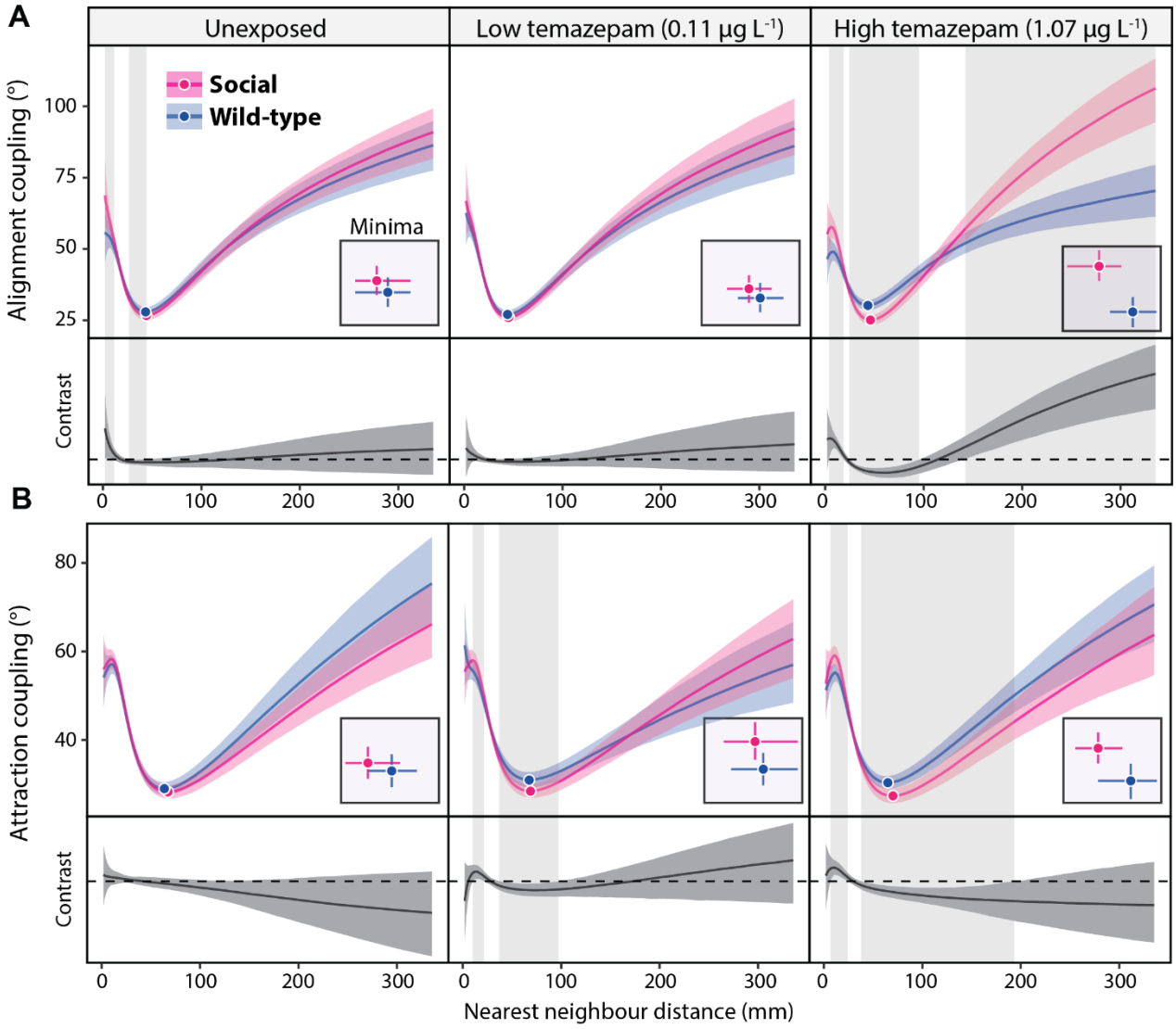

Fig. 4. Temazepam exposure modifies alignment and attraction coupling in a phenotype-dependent manner. **A, B**, Marginal estimates of alignment (**A**) and attraction (**B**) coupling as a function of nearest-neighbour distance under unexposed, low (0.11 µg L^{-1}), and high (1.07 µg L^{-1}) temazepam exposure. Within each treatment, groups are composed of either social (pink) or wild-type (blue) fish. Lines show marginal estimated medians, with shaded ribbons indicating 89% credible intervals (CrIs). Coloured circles denote posterior medians of draw-specific minima, representing peak alignment or attraction (where lower values indicate stronger coupling). Inset panels show the posterior medians and 89% CrIs for each selection line's minimum coupling angle and corresponding nearest-neighbour distance (note that axes are rescaled relative to the main plot). Lower panels show posterior contrasts (social-type – wild-type), with shaded ribbons indicating 89% CrIs and dashed horizontal lines denoting zero difference. Grey

vertical bands indicate neighbour-distance ranges where contrasts are credibly different (89% CrIs exclude zero).

DISCUSSION

Temazepam reshaped the collective behaviour of guppies in a phenotype-dependent manner. In wild-type groups, exposure generally reduced shoal coordination, with declines in polarisation, speed, and cohesion. Reduced group-level coordination was mirrored by weakened local alignment rules at the individual level. Social-type groups, by contrast, showed a distinct response profile, including stronger peak alignment under high exposure and no credible change in peak attraction. As a result, temazepam did not homogenise behavioural responses across groups, but instead amplified pre-existing differences between wild-type and social-type fish and elicited new points of divergence at both the collective and individual-interaction levels.

The effects of temazepam were pronounced during the early stages of group establishment, when individuals must rapidly detect, integrate, and respond to the movements of unfamiliar neighbours. This early phase is likely to be ecologically important in species with fission–fusion social dynamics, including *P. reticulata* (*22*), where group composition changes frequently and coordinated movement must be repeatedly re-established (*23*, *24*). If contaminant exposure delays or destabilises the formation of coordinated groups, even transient disruption could accumulate across repeated social reassembly events. Such effects may be particularly consequential because polarisation, swimming speed, and cohesion, jointly shape the ecological function of animal groups. Coordinated shoals can improve information transfer (*25*), increase energetic efficiency (*26*), enhance collective escape responses (*1*), and reduce predation risk through dilution and confusion effects (*27*). Disruption of these properties, therefore, has the potential to alter multiple axes of ecological performance, from predator avoidance and foraging to movement through heterogeneous environments.

By linking group-level responses to local interaction rules, our results provide a mechanistic explanation for how a neuroactive contaminant can scale from individual decision-making to emergent collective behaviour. In wild-type fish, temazepam exposure weakened alignment responses, meaning that exposed fish were less precise at matching the heading direction of their neighbours. This is consistent with the observed reductions in polarisation and shoal speed since polarisation is the group-level expression of shared

heading direction, and faster, more coherent shoal movement should emerge when individuals are more strongly aligned in their direction of travel. Thus, the breakdown of alignment at the local scale provides a plausible mechanism for the loss of coordinated motion at the group scale, consistent with models and empirical studies showing that collective movement emerges from local alignment and attraction rules among neighbours (*21*, *28*). Attraction rules were less consistently affected: low exposure weakened peak attraction in wild-type fish, while high exposure produced no credible peak change. This selective attraction response parallels the transient low-dose reduction in cohesion and the absence of a credible treatment effect on shoal size. Because attraction primarily serves to maintain spatial proximity among group members, the results indicate that temazepam disrupted directional coordination more consistently than it disrupted spatial organisation. These findings highlight local interaction rules as sensitive mechanistic endpoints for behavioural ecotoxicology, because they reveal how the effects of a pollutant on individual movement decisions can propagate into collective-level outcomes.

The divergent responses of wild-type and social-type groups indicate that the behavioural effects of temazepam depend on the underlying genotype and phenotype on which the contaminant acts. Social-type fish were generated by artificial selection for increased shoal polarisation and are known to differ from wild-type fish in collective movement, social interaction rules, and neurobiological traits associated with social responsiveness (*15*, *19*). In the present study, these baseline differences shaped contaminant sensitivity: high exposure weakened peak alignment in wild-type fish but strengthened it in social-type fish, whereas peak attraction remained stable across treatments in social-type fish. Consequently, exposure amplified rather than erased behavioural differences between lines. This result highlights that pollutants are unlikely to produce broadly uniform behavioural impairment across exposed populations. Instead, the same contaminant can generate different collective outcomes depending on pre-existing variation in the sensory, neural, and behavioural systems that generate social behaviour.

A likely explanation for this phenotype dependence is that temazepam acts on neural substrates that differ between line types. Social-type fish have a relatively enlarged optic tectum and thalamus, regions implicated in visual motion processing, sensory relay and the integration of information before motor decisions are made (*19*). These neuroanatomical differences are particularly relevant because coordinated shoaling depends on the rapid detection and integration of neighbours' positions and movements.

Social-type fish also show genetic and transcriptomic differences in neural development and synaptic function, including variation in *cdh13*, a sociability-linked gene associated with GABAergic functioning and brain activity (*29*). This is important because benzodiazepines are designed to modulate GABAergic signalling, and waterborne benzodiazepine exposure has been linked to altered brain GABA levels and GABA-receptor-mediated behavioural effects in fish (*30*). Moreover, benzodiazepines have been shown to preferentially accumulate in the brain of exposed fish (*31*), providing a direct route through which environmentally relevant exposure may affect neural circuits involved in behavioural regulation. Thus, phenotype-specific neural architecture and function provide a plausible substrate for the divergent collective responses observed here.

Divergent responses to temazepam may also reflect broader physiological differences between line-types. Selection on collective behaviour may have altered not only neural architecture, but also physiological traits that influence contaminant toxicokinetics, including uptake, tissue distribution, biotransformation, and elimination (*31*, *32*). Physiological divergence between line-types is plausible given the neuroanatomical differences associated with selection, because neural tissue is energetically demanding (*33*) and social behaviour can be linked to broader metabolic and gut–brain processes (*34*). Whether driven by neural circuitry, toxicokinetics, or both, the conclusion is the same: baseline social phenotype can shape how pollutant exposure translates into collective behavioural change. To extend the generality of these findings, future work should establish whether these effects extend to mixed-sex shoals, given there are both sex differences in physiology and shoaling behaviour (*20*), as well as documented sex-specific uptake and effects of other neuroactive pharmaceutical pollutants (*35*, *36*).

In conclusion, temazepam altered collective behaviour by reshaping the social interaction rules that generate coordinated motion, but these effects were dictated by baseline shoaling phenotype. Wild-type groups showed reduced coordination and weakened alignment under exposure, whereas social-type groups responded differently, including strengthened peak alignment under high exposure, leading to amplified divergence between phenotypes. These results demonstrate that neuroactive contaminants can have non-linear and phenotype-dependent effects on animal groups. This has important implications for environmental risk assessment, where behavioural endpoints are typically measured on isolated individuals and extrapolated to populations (*9*, *37*, *38*), yet it is clear that pollutant effects can depend on the interaction among contaminant, individual decision rules, and group phenotypes. Accounting for these levels of organisation will be

essential for predicting how neuroactive pollution alters animal behaviour in ecologically realistic settings.

MATERIALS AND METHODS

Animal ethics

All behavioural experiments and experimental procedures adhered to the animal ethics approvals at Stockholm University (Project numbers: 17362-2019 and 12211-2021).

Selection lines

We used 600 adult female guppies, the progeny of a unique experimental selection project for collective motion over three generations, as described in (*15*). In brief, six independent lines were maintained: three with directional selection for increased polarisation (social-type) and three control lines (wild-type), which were otherwise maintained in identical environmental and social conditions. For multigenerational selection, female guppies underwent an iterative sorting-and-mixing procedure by ranking groups (8 fish per group) according to median polarisation and mixing groups of similar rankings (**Fig. 3A**). Following 12 rounds of this procedure, repeatable variation in polarisation between groups was established (*15*). Breeding selection then targeted the upper 20% of female groups exhibiting the highest polarisation scores (*15*). Wild-type lines were handled and assayed similarly but were not subjected to selection. After three generations, social-type fish exhibited approximately 15% greater alignment, enhanced cohesion, and elevated swimming speed relative to wild-type (*15*). Female-only shoals were used because artificial selection for alignment was more strongly amplified in female groups, providing the clearest test of how baseline shoal phenotype shapes contaminant responses. Although sorting-and-mixing was applied at a group-level, this approach still constitutes powerful selection on the underlying individual-level behavioural variation (*39*), and thus, provides an opportunity to experimentally test how repeatable baseline differences in collective behaviour modulate the effects of neuroactive contaminants.

Exposure protocol

The experiment was conducted in eight staggered batches (5 replicates per treatment per batch), initiated on consecutive days so that all fish were exposed for exactly 10 d. All protocols were identical across batches. Fish were randomly assigned to one of three waterborne exposure treatments: (i) control (solvent-only), (ii) low-dose temazepam

(nominally 0.1 µg L^{-1}), or (iii) high-dose temazepam (nominally 1 µg L^{-1}). These concentrations fall within the range reported for aquatic surface water detections (mean = 0.021; range: <0.001–1.38 µg L^{-1}; (*13*)) and effluent detections (mean = 0.071, range: <0.001–1.86 µg L^{-1}; (*13*)). A closed static exposure was conducted in glass aquaria (10.8 L; *N* = 120 tanks), with fish housed in groups of five. Aquaria were dosed with 0.5 mL of concentrated temazepam stock solution (containing 1.215 µg and 12.150 µg for low and high treatments, respectively) in ethanol (≥98%) at the start of exposure, and re-dosed with 20% of the initial concentration on day five (see Supplementary methods for details). Temazepam (CAS: 846-50-4) was purchased from Sigma Aldrich (SKU: T8275; purity of ≥98 %). Control tanks received an equivalent volume of ethanol (i.e. 0.5 mL). Fish were held under a 12:12 h light:dark cycle, the average daily temperature was 24.0 °C (SD = 1.0, *N* = 152) and all pH levels were approximately 7 (detailed in Supplementary methods, Exposure protocol). The mean weight and length of fish used in the experiment were 185.8 mg (86.2–344.8 mg; *N* = 465, some fish were not weighed) and 21.44 mm (16.68–27.67 mm; *N* = 600), respectively. There was no mortality during the exposure period. Fish were fed once daily with commercial fish food flakes (JBL NovoBel).

Exposure verification

To verify treatment concentrations, a water sample was taken and analysed from half of the tanks during the 10 d exposure (*N* = 60 samples, 20 per exposure treatment). Sampling was balanced across exposure treatment (unexposed, low, and high) and the day of exposure (i.e. day 1–9), with at least two samples collected for each treatment on each day. Water samples were analysed using a liquid chromatography–tandem mass spectrometry approach following previously reported methods (*32*), as detailed in Supplementary methods, Exposure protocol. Temazepam concentrations closely matched nominal targets, deviating on average by <10% and remaining within environmentally realistic ranges (see Exposure protocol). Mean (± SD) concentrations were 0.11 ± 0.02 µg L^{-1} (*N* = 20; range 0.07–0.15) and 1.07 ± 0.18 µg L^{-1} (*N* = 20; range 0.63–1.30) for the low and high treatments, respectively (**Fig. 1A**). All control samples (*N* = 20) were below the limit of quantification (average limit of quantitation = 0.0026 µg L^{-1}; *N* = 60), confirming the absence of exposure.

Behavioural quantification and trajectory processing

Post-exposure, group behaviour was assessed in large circular open-field arenas (500 mm diameter) using shoals of five unfamiliar female fish from the same line and treatment. Fish were introduced into opaque acclimation chambers positioned equidistantly within

the arena and simultaneously released after a 5-min acclimation. Trials were recorded for 35 min at 25 fps, and tracking began 5 s post-release and continued for 34 min (further details of assay conditions are described in Supplementary methods, Behavioural assay). Nine video recordings were lost due to hardware malfunctions. The final processed analysis datasets contained 111 group trials (37 groups and 185 fish for each of the unexposed, low, and high treatments; 555 fish total). Individual fish trajectories were extracted from video recordings using idtracker.ai (version 6.0.3; (*14*), see also Supplementary methods, Trajectory processing and coordinate extraction).

All data processing and analyses were conducted in R (v. 4.3.2), and all traits were estimated from individual fish trajectories. Group-level metrics were derived from distance-based dynamic shoals calculated for each frame. More specifically, individuals were considered part of the same shoal when they were located within a 65 mm radius (approximately two body lengths), and, by extension, connected to others within this social radius (**Fig. 1E**). This threshold was selected based on established alignment response distances in these populations (*15*) and corresponded to the peak alignment and attraction distances estimated here (**Table S2**). From these dynamic groupings, we quantified shoal size (number of individuals; **Fig. 1E**), polarisation (mean directional alignment; **Fig. 1B**), shoal speed (magnitude of the mean shoal speed vector; mm s^{-1}; **Fig. 1C**), and cohesion (mean nearest-neighbour distance; **Fig. 1D**). Leadership turnover was estimated as the number of leadership changes divided by the number of valid leadership opportunities (**Fig. 1F**). Leaders were defined as the most anterior individual relative to group heading (±90° of orientation) when shoal speed exceeded 20 mm s^{-1}. All traits were averaged across minute-level bins (min 1–34). Full computational details are provided in the Supplementary Methods, Trajectory processing and coordinate extraction.

Individual-level alignment and attraction interactions (i.e. local interaction rules) were quantified using a dyad-based burst–coast framework following Calovi *et al.* (*40*). Guppies swim using discrete acceleration events ("bursts") during which rapid changes in heading occur, followed by passive deceleration phases ("glides"). We defined movement decisions as the change in heading at burst onset and related these turning responses to the relative position (attraction coupling) and orientation of the nearest neighbour (alignment coupling). Alignment coupling captures turning to reduce angular disparity in heading, whereas attraction coupling captures turning toward a neighbour's spatial position (Fig. 2B). Full computational details are provided in the Supplementary Methods, Trajectory processing and coordinate extraction.

Statistical analyses

Statistical analyses were performed using Bayesian models implemented in *brms* (*41*) with the *cmdstanr* backend. Group-level responses were analysed using hierarchical generalised additive models to capture non-linear temporal dynamics. Each model included a global smooth of time and smooth deviations for exposure treatment, selection line, and their interaction, alongside parametric treatment and selection effects, trial round, and random intercepts for trial nested within batch. All smooths were fitted with thin-plate regression splines with 5 to 10 knots, with the number of knots and distributional assumptions of the models tailored to each parameter (see Supplementary Methods for details). Default prior structures were inspected and calibrated using prior-predictive simulation. Weakly to mildly regularising priors were specified for the group-level models, with intercept priors centred on observed means on the appropriate response or link scale. The burst-frequency model retained the brms defaults, while the multivariate alignment–attraction model used normal(0, 3) priors for population-level coefficients in both responses. Full prior specifications are provided in the Supplementary Methods and analysis scripts.

Individual-level total burst frequency was modelled with exposure, selection, and their interaction as fixed effects and trial as a random intercept. Alignment and attraction coupling components were modelled jointly in a multivariate framework, allowing shared predictors while estimating separate smooth functions and variance structures for each response. These models included smooth terms for nearest-neighbour distance and within-trial time, as well as interaction-specific smooth deviations of neighbour distance, with random intercepts for trial and fish identity. Again, all smooths were fitted with 3- to 6-knot thin-plate regression splines, with the number of knots tailored to the splines.

All models were fitted with four Markov chains using conservative tuning parameters (adapt_delta ≥ 0.95, max_treedepth = 12). Convergence was confirmed using trace inspection, effective sample sizes, and $\hat{R} < 1.01$. Model adequacy was assessed using posterior predictive checks. Estimated marginal means and contrasts were derived from posterior draws, and inference was based on posterior medians and 89% credible intervals, with effects considered meaningful when intervals did not overlap zero.

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