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Risk-Avoiding Strategies in Rotifer Diapause-Related Traits in Response to Environmental Unpredictability

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ABSTRACT

1. Unpredictable environments often drive organisms to adopt risk-avoiding strategies to secure fitness over time. Among life-history traits, the timing of leaving dormancy is frequently studied; however, it requires careful examination, incorporating a multi-component, non-stationary and organism-specific perception of environmental predictability.
2. Here, we investigated the interplay of diapause-related traits in *Brachionus plicatilis*, a cyclically parthenogenetic rotifer species, from natural populations inhabiting ponds that cover a gradient of predictability regarding hydrological conditions.
3. By reanalysing the dataset published by Franch-Gras and co-workers in 2017 on diapause-related traits from wild populations of this species in nine shallow, non-permanent, brackish water bodies, we linked these traits to environmental parameters derived from Landsat 5 and 7 multispectral images. These non-stationary parameters—average hydroperiod length, coefficient of variation of hydroperiod length and Colwell's index for predictability—were analysed to assess their influence on each diapause-related trait.
4. Our findings revealed that rotifers primarily respond to uncertainty by adopting conservative bet-hedging strategies, ensuring the early production of diapausing eggs. Additionally, hatching-related traits exhibit potential trade-offs, responding to distinct components of environmental unpredictability, such as that occurring within and among growing seasons.
5. This study underscores the importance of incorporating multiple components of predictability and their interactions when evaluating bet-hedging strategies.

1 | Introduction

Organisms living in time-varying environments have to match the timing of their life history events, such as birth, growth or reproduction, with the occurrence of favourable conditions to maximise survival and reproductive success. This becomes particularly challenging when organisms lack accurate information about the timing of these conditions; that is, when their environments fluctuate unpredictably. In such environments, theory predicts the evolution of bet-hedging strategies (Cohen 1966; Hopper 2014; Botero et al. 2014). These are risk-avoiding strategies that reduce the time variation of fitness at the expense of a reduction in the arithmetic mean of fitness, resulting in an increase in its geometric mean (Slatkin 1974; Philippi and Seger 1989). Two main types of bet hedging are distinguished, namely conservative and diversified (Philippi and Seger 1989). Under conservative bet hedging, risk is minimised when a genotype adopts a single, generalist phenotype that works reasonably well across all possible environmental conditions. In contrast, diversified bet hedging occurs when a single genotype produces multiple phenotypes among its offspring to cope with future unpredictable conditions (Simons 2011). Bet hedging, in either of the two types, can be studied in a variety of life history traits, but in time-varying environments, it would be expected

to be particularly associated with dormancy, given its key role in coping with unfavourable conditions. Specifically, the timing of leaving dormancy is one of the most theoretically addressed life-history traits in the context of bet hedging (Cohen 1966; Venable 1989; Spencer et al. 2001; Ten Brink et al. 2020). The asynchronous exit from dormancy, manifested as intermediate fractions of reactivation—such as seed germination or egg hatching—of the dormant stages produced by an individual or genotype, is postulated to buffer the risk of complete reproductive failure in the event of unexpectedly unfavourable environmental conditions. Despite the substantial theoretical advancement in understanding bet hedging in the timing of leaving dormancy, empirical evidence of its occurrence in the natural world remains limited. Most evidence arises from studies of seed germination in plants (e.g., Gremer and Venable 2014) and with comparatively lesser strength of evidence from studies on animals. Notable exceptions include research on the hatching fraction of diapausing eggs in aquatic invertebrates (e.g., Tarazona et al. 2017; Pinceel et al. 2021). While most studies have focused on this diversification trait, it is important to note that conservative bet-hedging strategies, potentially associated with the timing at which dormant stages are produced, could be an alternative response to face environmental unpredictability (see Simons and Johnston 2003; Tarazona et al. 2017). These studies, however, are even rarer in the literature (for review, see Simons 2011).

Defining predictability presents several significant challenges, including its quantification. Numerous methods for assessing the degree of predictable environmental variability have been proposed in the scientific literature (Sabo and Post 2008; Tonkin et al. 2017). Among these methods, Colwell's index (1974) uses information theory to measure predictability through the sum of two components: constancy, which reflects the degree to which a system remains in the same state over time, and contingency, which captures the repeatability of the variation patterns. Other approaches, based on statistical descriptors of variation (e.g., the standard deviation or the coefficient of variation; Beissinger and Gibbs 1993) or spectral analyses (Torrence and Compo 1998) have also been employed. These approaches focus on stability and dominant frequency decomposition, respectively. The above-mentioned methods are often applied individually, neglecting the possibility that environmental predictability could be a multi-component phenomenon. If so, this practice would limit a comprehensive understanding of the full spectrum of dimensions that may constitute the predictability of any ecological factor. Another major challenge lies in the own variation of environmental predictability. Many ecological factors show changing features at the time scale typical in ecological studies. Hence, the degree of predictability of environmental fluctuations might also be subject to temporal variability (Meehl et al. 2000; Sabo and Post 2008; Doblas-Reyes et al. 2013; Young et al. 2020). This variability introduces uncertainty in selecting an appropriate time frame to compute environmental predictability. Although it may seem that longer observation periods make the estimation of the degree of predictability more reliable, that estimation might be integrating too long, heterogeneous periods, while the organisms might have evolved in response to the nearest conditions. This issue is closely tied to a third challenge: how the focal organism perceives environmental variation, that is, whether it is more or less predictable for the focal organism. The evolutionary impact of environmental changes depends on the generation time and lifespan of organisms (Turner et al. 1998; Lytle 2001; Chevin et al. 2010; Bernhardt et al. 2020). When the temporal scale of variation of these traits matches the frequency of environmental changes, a strong evolutionary response is expected. Conversely, an organism with a short lifespan in an environment with low-frequency alternations may never experience those changes within its lifetime and thus will not be subject to that selection pressure (Iwasa and Levin 1995; Lytle and Poff 2004). Therefore, characterising the predictability of any habitat requires consideration of the perspective of the organism of interest, as well as a suite of relevant traits taken simultaneously, since focusing on a single trait may not capture the full adaptive response (Franch-Gras, García-Roger, Serra, and Carmona 2017; Franch-Gras et al. 2019; Bernhardt et al. 2020; Musters et al. 2023).

In this study, we examined several potential bet-hedging traits in relation to several components of predictability using cyclically parthenogenetic rotifers as a model study organism (Serra et al. 2019). These facultative sexual aquatic microinvertebrates typically inhabit water bodies suitable for population growth only temporarily (García-Roger et al. 2006). Active populations recolonize the water column during the so-called planktonic growing season. To survive between growing seasons, rotifers produce diapausing eggs, a dormant stage (Gilbert 1974, 2007). At the onset of the growing season, diapausing eggs from the sediment bank hatch into asexual (amictic) females, which produce subitaneous eggs via ameiotic parthenogenesis. These eggs hatch into genetically identical daughters, enabling the proliferation of clonal lineages over multiple generations. Sexual reproduction is induced by environmental cues (e.g., Gilbert 1974; Schröder 2005) that trigger asexual females to produce sexual (mictic) daughters as a fraction of their offspring, so that asexual reproduction continues to occur alongside sexual reproduction during this phase. Sexual females produce meiotic haploid eggs, which may develop into males if unfertilized, or into diapausing eggs if fertilised. The diapausing eggs settle in the sediment for varying periods, enabling rotifer genotypes to survive desiccation and other adverse conditions (Ricci 2001; Schröder 2005). Upon the return of favourable conditions, some of these diapausing eggs hatch, recolonizing the water column and starting a new growing season. Thus, the persistence of a given rotifer genotype into the following growing season depends on its diapausing eggs and the adjustment of their traits to the length and predictability of the hydroperiods characterising the water body (Smith and Snell 2012; Franch-Gras, García-Roger, Serra, and Carmona 2017; Tarazona et al. 2020). Hydroperiod length defines the position of a pond along an ephemerality-permanence gradient, which is a key selective factor of any water body, independently of its predictability (Smith and Snell 2012). Worth noting, hydrological characteristics may act as selective pressures directly or indirectly through associated ecological factors such as salinity, food availability or predator density. The degree of predictability, which depends on whether hydroperiods have a regular or random length and distribution, could in the latter case select for bet-hedging traits associated with diapause. Interestingly, Franch-Gras, García-Roger, Serra, and Carmona (2017) addressed the relationship between a particular component of hydroperiod predictability and several diapause-related

traits in wild populations of the rotifer *Brachionus plicatilis*. Franch-Gras and his co-authors conceived predictability in relation to the annual pattern for the presence of water in the habitat of a population, as inferred from satellite observations (Franch-Gras, García-Roger, Franch, et al. 2017). These authors showed that rotifer genotypes from unpredictable water bodies initiated sexual reproduction earlier, likely as a way of ensuring diapausing egg production before an unexpected end of the growing season. Although Franch-Gras, García-Roger, Serra, and Carmona (2017) also reported intermediate hatching fractions of diapausing eggs, these were independent of the degree of hydroperiod predictability of their habitat of origin, thus contradicting theoretical predictions.

Building on the arguments described above regarding the challenges in finding adequate metrics to quantify the predictability as perceived, in this case, by rotifers, and trying to expand the suite of traits associated with diapause whose expression can be framed as a bet-hedging strategy, we propose here a revisitation of the study system and raw data of Franch-Gras, García-Roger, Serra, and Carmona (2017), available to the scientific community in Dryad (https://doi.org/10.5061/dryad.5vq2h). To this end, in the present study, we refined the characterisation of environmental predictability in different ways. First, we used an improved methodology for assessing the presence of water in the studied ponds and lakes, which allowed us to increase the number of valid observations from the satellite-acquired scenes. Second, we varied the approach to characterise hydroperiod patterns. Besides Colwell's index, which attends to the predictability of the presence of water in a water body during the annual cycle, we used the hydroperiod length as the timespan with continuous presence of water, as well as the variation of that length. Regarding diapause-related traits, we added traits after elaborating the database collected by these authors (Franch-Gras, García-Roger, Serra, and Carmona 2017). The life-history traits we examined were: (1) the population density and (2) the time for sex initiation, both of which are related to the timing of diapausing egg production; and (3) the hatching time of diapausing eggs, (4) its variation, and (5) the fraction of diapausing eggs that hatch, all three associated with the exit from diapause. Our focus is on ultimate, evolutionary causes, rather than proximate mechanistic ones. Regarding the latter, existing physiological knowledge on rotifers provides putative mechanisms that might explain how the response of diapause-related traits can be adjusted to the expected optimum under a given hydroperiod regime. This would be the case of populations having different threshold densities for the initiation of sexual reproduction, to match their different statistical distribution of hydroperiod length.

Specifically, we proceeded with the following hypotheses: (a) delayed diapausing egg production will be selected under longer hydroperiod regimes. This delay would allow for prolonged clonal proliferation, leading to larger final population abundance and, consequently, a greater production of diapausing eggs. On the other hand, (b) an earlier production of diapausing eggs will be expected in unpredictable habitats to ensure new recruitment of diapausing eggs and clone survival even during the shortest hydroperiods that might occur. With regard to exiting diapause, (c) an earlier timing of diapausing egg hatching will be selected under shorter hydroperiods. Ephemerality is an abiotic factor that may exert a very intense selection pressure driving diapausing eggs to hatch as soon as possible in the growing season. Otherwise, rotifer clones may fail to proliferate and complete their life cycle in a very short growing season. In contrast, longer hydroperiods would not exert such an intense selective pressure for the timing of hatching, allowing for potential mutation accumulation that could result in some delay in the time of hatching. Although other forces, such as competition among rotifer genotypes for exploiting the growing season, could also be selecting for early hatching, here we hypothesise that abiotic factors, if taken to the extreme of ephemerality, are prevalent. (d) Greater variation in hatching time will be selected in clones inhabiting unpredictable habitats to spread the risk of hatching during unexpected unfavourable periods occurring early within a growing season. Also, in unpredictable habitats (e) lower diapausing egg hatching fractions would be selected for to hedge the uncertainty of hatching during an adverse growing season, such as those with excessively short hydroperiods.

Given that hydrological regimes are expected to show a secular shift, especially as a result of global change, we examined whether the relationship between hydroperiod length, predictability, and the studied diapause-related traits varies with the length of the time period studied. Finally, we investigated the potential for diapause-related trait syndromes to address varying levels of environmental predictability, including possible trade-offs between single traits. To this end, we examined multiple traits concurrently to explore their interconnections and assess adaptive strategies in response to environmental unpredictability.

2 | Material and Methods

2.1 | Study Populations and Habitat Characterisation

We reanalysed the raw database of diapause-related traits collected by Lluís Franch-Gras and co-workers (Franch-Gras, García-Roger, Serra, and Carmona 2017) on nine wild populations of the rotifer *B. plicatilis*. These populations inhabit shallow, endorheic, non-permanent, brackish ponds and lakes in a small area of the Eastern Iberian Peninsula (Figure 1). These water bodies are close to each other, but they are not connected by superficial flow. The area of the smallest convex polygon that encloses all studied water bodies is 106 km^2, and the distance between the farthest water bodies is 16 km. The studied water bodies differ in size and flooding pattern (Franch-Gras, García-Roger, Serra, and Carmona 2017; Franch-Gras, García-Roger, Franch, et al. 2017). The dataset of diapause-related traits corresponds to a sampling campaign of the diapausing egg banks of the *B. plicatilis* population in the nine studied localities (ponds and lakes) performed in 2013 (see below).

For the hydrological characterisation of the habitats of the nine studied populations, we gathered a timeseries (period 1984–2013) of 656 satellite scenes from Landsat missions 5 and 7. Each of these scenes includes all our water bodies and corresponds to path 199 and row 33 (approximate scene size equal to 170 km north–south by 183 km east–west). The

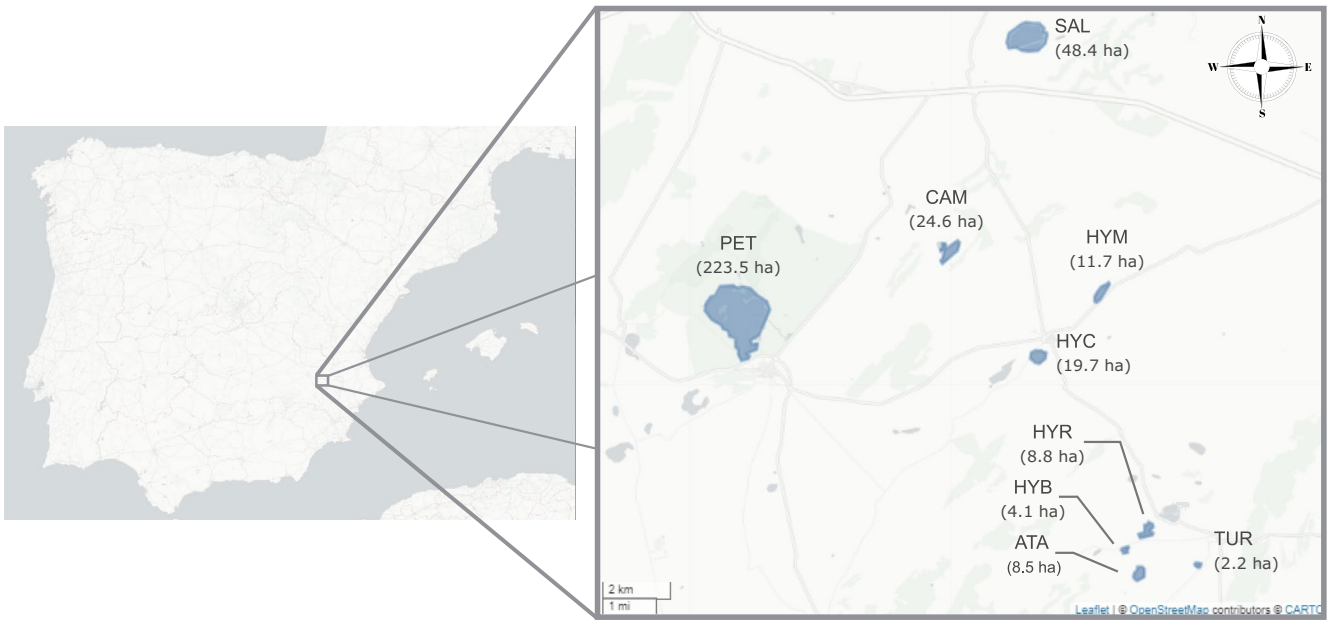

FIGURE 1 | Location (coordinates of NW corner: 38°55′29″ N 1°35′31″ W; SE corner: 38°45′43″ N 1°20′53″ W; Datum: ED50) of the nine studied water bodies (light blue) in the Eastern Iberian Peninsula. ATA, Atalaya de los Ojicos; CAM, La Campana; HYB, Hoya Yerba; HYC, Hoya Chica; HYM, Hoya del Monte; HYR, Hoya Rasa; PET, Pétrola; SAL, Salobralejo; TUR, Hoya Turnera. The surfaces indicate the maximum inundation area observed throughout the entire series of available years (1984–2013). SIGPAC Ministry of Agriculture, Fisheries and Food, Government of Spain.

satellite scenes were downloaded through the United States Geological Survey's (USGS) Earth Explorer API (https://earthexplorer.usgs.gov/). These had a spatial resolution of 900 m^2 and a temporal frequency of 1/16 d^{-1}. Satellite scenes were processed to discern the presence or absence of water in the studied water bodies. With this aim, firstly, for each water body we defined an extent by delineating a rectangle circumscribing as closely as possible the water body location, which was determined in Franch-Gras, García-Roger, Franch, et al. (2017). This extent allowed us to analyse water body-specific scenes, hereafter called simply as 'scenes', as opposed to the 'satellite scenes' referred to above. Secondly, we applied the Modified Normalized Difference Water Index (MNDWI; Xu 2006) in order to identify candidate pixels indicating water. Thirdly, we applied Ormeci and Ekercin (2007) filter to discriminate salt crusts—commonly observed in the studied water bodies when dry—from actual water. Following Franch-Gras, García-Roger, Franch, et al. (2017), the presence of a single water pixel within a water body extent was sufficient to classify it as containing water. However, surface water pixels can still be mistaken because of the presence of clouds. Franch-Gras and co-workers dealt with this problem by removing any satellite scene with a cloud, as estimated using the default USGS' Earth Explorer cloud filters. We addressed this difficulty in a different way. We considered an additional rectangle enclosing each extent, the sides of the former being at 75 m from the sides of the latter. The resulting strip (a rectangular frame) between the two rectangles was used as a cloud-check area around each water body extent. No strip associated with a water body overlapped with other nearby water bodies. Because a water pixel in a strip should correspond to clouds, if no water pixels were detected in the cloud-check area of a water body, the scene was assumed to be cloud-free and retained for further analyses. Otherwise, the scene was discarded. This procedure yielded 446 to 571 putatively cloud-free scenes per water body (average: 527 scenes per water body; Table S1). For all water bodies, the number of retained scenes surpassed the number obtained using the default USGS' Earth Explorer cloud filters (423 scenes; in Franch-Gras, García-Roger, Serra, and Carmona 2017).

The selected cloud-free scenes were subsequently used to compute three environmental parameters. (1) The average hydroperiod length for each water body was calculated as the average duration (in days) of consecutive observations showing uninterrupted water presence during the focal period. To assess habitat predictability, we computed (2) the coefficient of variation of the hydroperiod length (henceforth, CVH; Weber et al. 2004), which is a dimensionless inverse measure of the constancy in hydroperiod length of a given water body. As an additional predictability metric, (3) Colwell's index was calculated, providing a complementary perspective. Extensively used to characterise environmental variation (Stearns 1981; Sabo and Post 2008; Tonkin et al. 2017), Colwell's index is based on a discretisation of time—treating it as a categorical rather than a quantitative factor or an ordinal variable—and a classification of environmental states. In the resulting two-way time × state table, the cells are the counts. From this table, contingency and constancy, the two components of predictability (Colwell 1974), can be computed. Colwell's index varies from 0 to 1, with higher values indicating greater habitat predictability. In our application, we followed the approach described in Franch-Gras, García-Roger, Serra, and Carmona (2017); Franch-Gras, García-Roger, Franch, et al. (2017), where unpredictability relates to the equiprobability of two states (flooded vs. desiccated) within a given calendar month. As a result, the index describes the predictability of the interannual pattern of the presence of water on a monthly scale. Worth noting, CVH and Colwell's index might be sensitive each to a different type of uncertainty. CVH does

not account explicitly for the time in the annual cycle when a hydroperiod occurs, although it might be affected by the propensity of flooding in a specific period of the year. By contrast, Colwell's index does not account for the time course of the events, as the order of the discretised times of the year has no effect on this metric.

2.2 | Characterisation of Diapause-Related Traits at Clonal and Population Levels

The experiments for building the raw database of diapause-related traits of Franch-Gras and coworkers are described in detail in Franch-Gras, García-Roger, Serra, and Carmona (2017). Briefly, rotifer clones were founded in the laboratory from diapausing eggs sampled in September 2013 from the uppermost 10 cm of sediment of each water body, where the largest fraction of viable eggs is located. Diapausing eggs were isolated (Gómez and Carvalho 2000) and induced to hatch (see García-Roger et al. 2006), and the resulting clones were genetically identified by PCR-RFLP as *B. plicatilis* (Campillo et al. 2005). This resulted in a collection of 30 clones of each population. In a first experiment, Franch-Gras, García-Roger, Serra, andCarmona (2017) estimated the propensity for sexual reproduction in these clones by performing sex self-induction bioassays (9 populations × 30 clones × 3 replicates = 810 bioassays) following the procedure described in Carmona et al. (2009) with minor modifications. Sex self-induction bioassays are based on the fact that *s*exual reproduction is density-dependent in the genus *Brachionus*, as it is triggered by a threshold concentration of an infochemical produced by the rotifers themselves and released into the medium (Carmona et al. 1993; Snell et al. 2006; Stelzer and Snell 2003, 2006). Prior to the bioassays, a pre-experimental phase involving three asexual generations was carried out with the objective of controlling for maternal effects. The bioassays of sex self-induction consisted of monitoring cultures initiated with a single neonate female, checked at 12-h intervals, and allowed to proliferate until the appearance of males. Once a male was observed in a culture, time and female density were recorded. This made it possible to measure for each replicate both the density and the time for sex initiation as proxies for the timing of diapausing egg production; the earlier or the lower the density at which the male appears, the earlier the initiation of sexual reproduction, and therefore, the production of diapausing eggs.

In a second experiment, Franch-Gras, García-Roger, Serra, and Carmona (2017) measured patterns of diapausing egg hatching. Diapausing eggs were produced in large quantities from mass monoclonal cultures, then isolated individually, subjected to standard hatching conditions (García-Roger et al. 2006), and checked every 24 h over a 28-day period to detect hatchlings. A total of 4605 diapausing eggs, corresponding to 9 populations × 5–6 clones per population × 96 diapausing eggs per clone, were monitored, which made it possible to estimate for each clone the average hatching time of the diapausing eggs, the within-clone variation in hatching time using the interquartile range of single egg hatching time (hereafter, IQR-hatching time), and the hatching fraction. The records made on each single egg for the hatching time and whether the diapausing egg hatched or not were retained for statistical testing.

2.3 | Data Analysis

We scrutinised the diapause-related trait raw database to identify outliers and exclude cases with limited replicated observations per clone. Outlier removal resulted in the retention of 808 out of 810 observations for the density and the time for sex initiation. For the hatching time of diapausing eggs, the IQR-hatching time and the hatching fraction, we excluded 10 clones with fewer than 30 monitored diapausing eggs. Consequently, 4402 diapausing eggs from 76 different clones were included in the analysis.

Statistical significance of the effects of the environmental parameters on each diapause-related trait was assessed using unidirectional tests, in accordance with our a priori predictions. Density for sex initiation was analysed using a generalised linear mixed model (GLMM) with a Poisson distribution of errors and log as link function. Time for sex initiation was not tested, as it resulted to be highly correlated with the density for sex initiation. The hatching time of diapausing eggs was modelled with a Cox proportional hazards mixed-effect model, which is a time-to-event model, with the day of hatching as the time-to-event response variable. The variation in hatching time (IQR-hatching time) was analysed using a linear mixed model (LMM). The hatching fraction was analysed on an egg-by-egg basis as a binary response, assuming a binomial distribution for the error in a GLMM with logit link function. In all models, the environmental parameters—average hydroperiod length, CVH and Colwell's index—were included as covariates. Population and clone nested within population were treated as random-effect factors, except for the LMM on variation in hatching time, which included only population as a random effect because only a single IQR-hatching time can be computed for each clone. Reduced models were developed to evaluate the effect of both population and clone as random effects on each diapause-related trait using likelihood ratio tests (LRT's). To illustrate the specific effect of each of the environmental parameters as posited in our hypotheses, partial residuals were calculated from linear models (LM) fitting the population averages of the diapause-related traits to the other two environmental parameters (Cook and Weisberg 1982). These partial residuals were finally regressed against the environmental parameter of interest. Note that although there were five main hypotheses relating diapause traits to hydroperiod and predictability (see Section 1), the latter was assessed using two predictability metrics, the effect of which was assessed individually. This brings the total number of relationships tested to eight.

Environmental parameters were calculated for decade intervals (1984–1993, 1994–2003 and 2004–2013), which allowed us to explore their variation throughout the entire period. As some variation was observed, the 2004–2013 decade was chosen in our analysis. The rationale here is that the rotifer populations were sampled in 2013, and the studied traits have the potential to evolve rapidly (Tarazona et al. 2017), the expectation is that our focal traits were shaped by the environment in the recent past. We further analysed the sensitivity of our analysis using periods shorter than a decade, namely 5 years (2009–2013) and 7 years (2007–2013).

As clues for environmentally related trait syndromes, we explored associations of diapause-related traits and their relationship with the environmental parameters. A redundancy analysis (RDA) was applied to population averages of diapause-related traits and environmental parameters, the latter regarded as predictors. Additionally, Pearson's correlation coefficients between the diapause-related traits and their statistical significance (unidirectional tests) were computed.

All analyses were performed in R v. 4.3.2 (R Core Team 2023). GLMMs and LMM were applied using, respectively, the *glmer* and *lmer* functions from the lme4 package (Bates et al. 2015). Cox proportional hazards mixed-effect model on the timing of hatching was performed using the *coxme* function from the coxme package (Therneau 2024). Partial residual analyses and associated LMs were computed using the *crPlots* function from the car package (Fox and Weisberg 2019). RDA was computed using the *rda* function in the vegan package (Oksanen et al. 2022).

3 | Results

We observed variation in the environmental parameters when computed for three consecutive 10-year spans (Figure 2A–C; Table S2). The between-decade correlation analysis (Figure 2D) showed that all the environmental parameters varied in a similar way across ponds in the first two decades, particularly in the average length of the hydroperiod and its coefficient of variation (CVH). The correlation between the decades 1994–2003 and 2003–2013 was low, except for the average hydroperiod length. Nearly all the water bodies shifted to longer average hydroperiod lengths in the last decade (Figure 2A). This regularity was remarkable in SAL and ATA, which showed average hydroperiod length values 35% higher than the values computed for the three decades (Figure 2A). These two ponds show relatively low CVHs (Figure 2B) and high values for Colwell's index (Figure 2C). Contrarily, the increase in the average length of hydroperiod in HYB (ca. 15%) was associated with a decrease in Colwell's predictability (Figure 2C). No noticeable discrepancies were observed in the relationships between the traits and environmental parameters for different timespans within the 2003–2013 decade (i.e., last 5, 7 and 10 years; Table S3). Therefore, the latter 10-year period was selected due to its larger sample size.

The effect of the environmental parameters, as estimated for the most recent studied decade, on the diapause-related traits is shown in Table 1 and Figure 3. The density for sex initiation was positively affected by the average hydroperiod length (Table 1; Figure 3B) and negatively affected by CVH (Table 1; Figure 3C). Albeit marginally significant, we found the expected relationship of IQR-hatching time with Colwell's predictability index (negative; Figure 3F); this trend suggests that higher variation in hatching time is associated with Colwell's unpredictability.

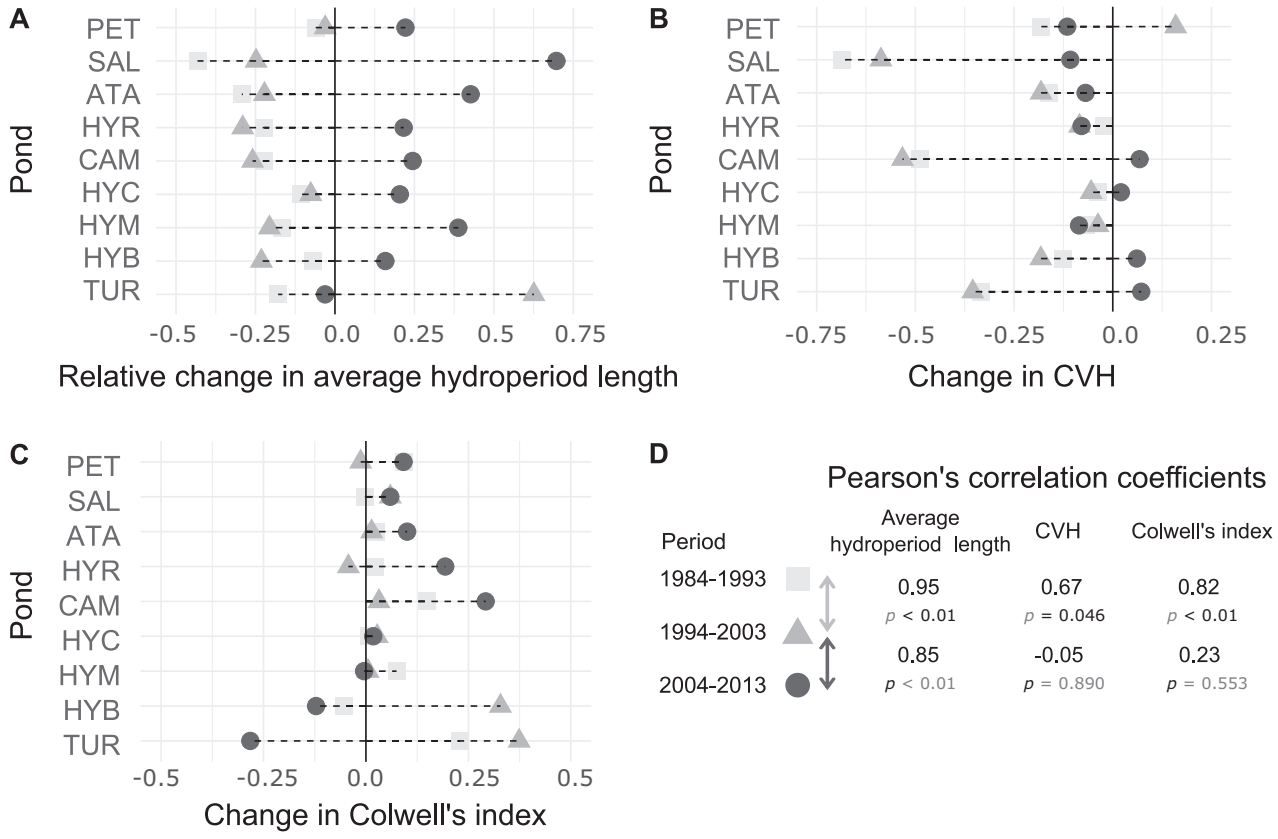

FIGURE 2 | Variation in environmental parameters in the studied pond. A, B and C along the decades 1984–1993, 1994–2003 and 2004–2013. The differences between the value for each of the three decades and the value computed for the three decades as a whole (1984–2013; pooled value) are shown. (A) Relative change in average hydroperiod length; values are the difference between each decade's mean and the pooled mean (1984–2013), divided by the pooled mean, which standardises across ponds of different sizes. (B) Coefficient of variation of hydroperiod length (CVH). (C) Colwell's index. (D) Pearson's correlation coefficients between the contiguous decades across ponds ($n = 9$; p values are shown).

TABLE 1 | Statistical analysis for the effects of environmental parameters on the density for sex initiation (after a generalised linear mixed model, GLMM), diapausing egg hatching time (after a Cox proportional hazard mixed-effect model), the variation in hatching time (IQR-hatching time; after a linear mixed model, LMM) and the hatching fraction (after a GLMM).

	Density for sex initiation			Hatching time			IQR-hatching time			Hatching fraction		
Effect	**RST**	***z* value**	***p***	**RST**	***z* value**	***p***	**RST**	***t* value**	***p***	**RST**	***z* value**	***p***
Average hydroperiod length	+	4.15	<0.001	+	−1.65	0.951	+/−	1.09	0.281	+/−	1.45	0.147
CVH	−	−4.11	<0.001	+/−	0.08	0.940	+	0.13	0.448	−	−0.57	0.283
Colwell's index	+	−0.46	0.324	+/−	1.52	0.128	−	−1.35	0.091	+	−3.23	0.999

Note: RST (Relationship statistically tested) is the predicted direction of the relationship that was statistically tested: +, positive effect hypothesised (one-tailed test); −, negative effect hypothesised (one-tailed test); +/−, no directional hypothesis (two-tailed test). Shaded cells highlight tests with a unidirectional hypothesis.

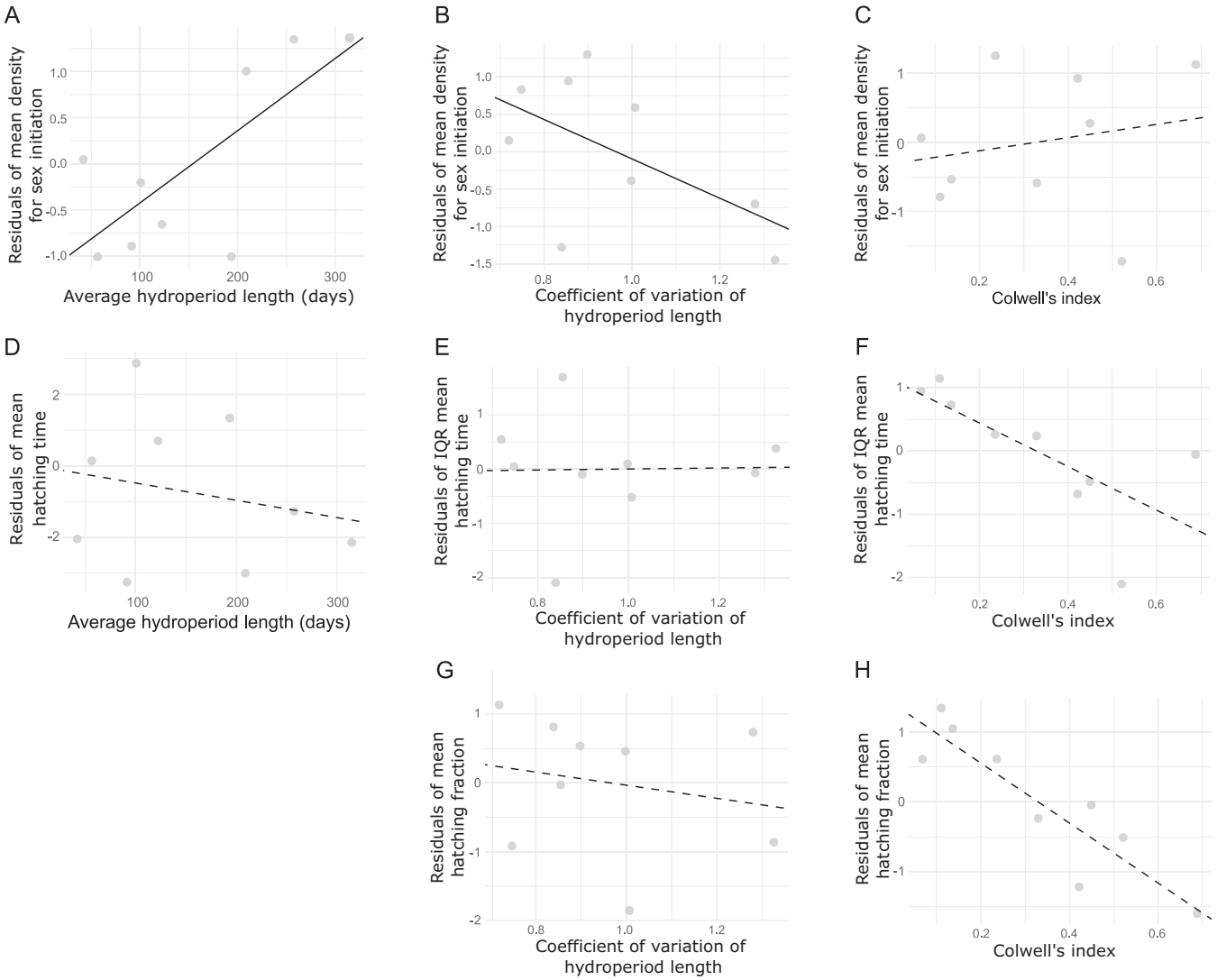

FIGURE 3 | Relationship between those diapause-related traits and environmental parameters for which specific hypotheses were stated and tested. Values of the traits are residuals after accounting for the other covariates in the corresponding statistical model. Linear fittings after least-square linear regression are shown. Solid lines indicate statistically significant regressions; dotted lines indicate non-significant regressions included to show trends.

Finally, we observed a negative trend between hatching fraction and CVH (Figure 3G). Our data revealed statistically significant differences among populations and among clones within populations in all traits (p value <0.05 for all LRTs) except for among-population differences in hatching time, which were marginally significant (p value $=0.053$).

The RDA for the relationships between the combinations of diapause-related traits per population and environmental parameters accounted for 60.3% of the total variance in our dataset. This variance was mainly accumulated by the first axis (98.6%), which is strongly and negatively related to average hydroperiod length (Pearson's $r = -0.656$) and Colwell's index (Pearson's $r = -0.572$). RDA1 scores for the density and time for sex initiation were negative and associated with average hydroperiod length (Figure 4A). Hatching time was mainly positively associated with Colwell's predictability index, while the hatching fraction and IQR-hatching time did not strongly associate with any environmental parameter (Figure 4B). Pair-wise correlations of diapause-related traits are shown in Figure 4C. We found a statistically significant relationship between the time and density for sex initiation (Pearson's $r = 0.797$, p value $= 0.01$). Although not statistically significant, hatching time showed negative relationships with both IQR-hatching time (Pearson's $r = -0.337$, p value $= 0.374$) and hatching fraction (Pearson's $r = -0.54$, p value $= 0.133$).

4 | Discussion

This study afforded three identified issues that emerge when investigating the adaptive responses of organisms to environmental predictability: specifically, how to conceive, decompose and quantify predictability, how to account for its variation, and on which trait(s) the studies should be focused. These issues are inherently dependent on the focal organism, in this case rotifers, one of the organisms in which studies on adaptation to environmental unpredictability have been addressed to the greatest extent.

Regarding the first issue of conceiving predictability of the hydroperiod in the rotifer habitat, taken as a proxy for the environmental predictability perceived by our rotifer populations, we approached it as a two-fold concept, encompassing the variation in the hydroperiod length and the interannual variation in their monthly distribution. Both components were estimated quantitatively, the former using the coefficient of variation (CVH) and the latter using Colwell's index on data of presence or absence of water. The low

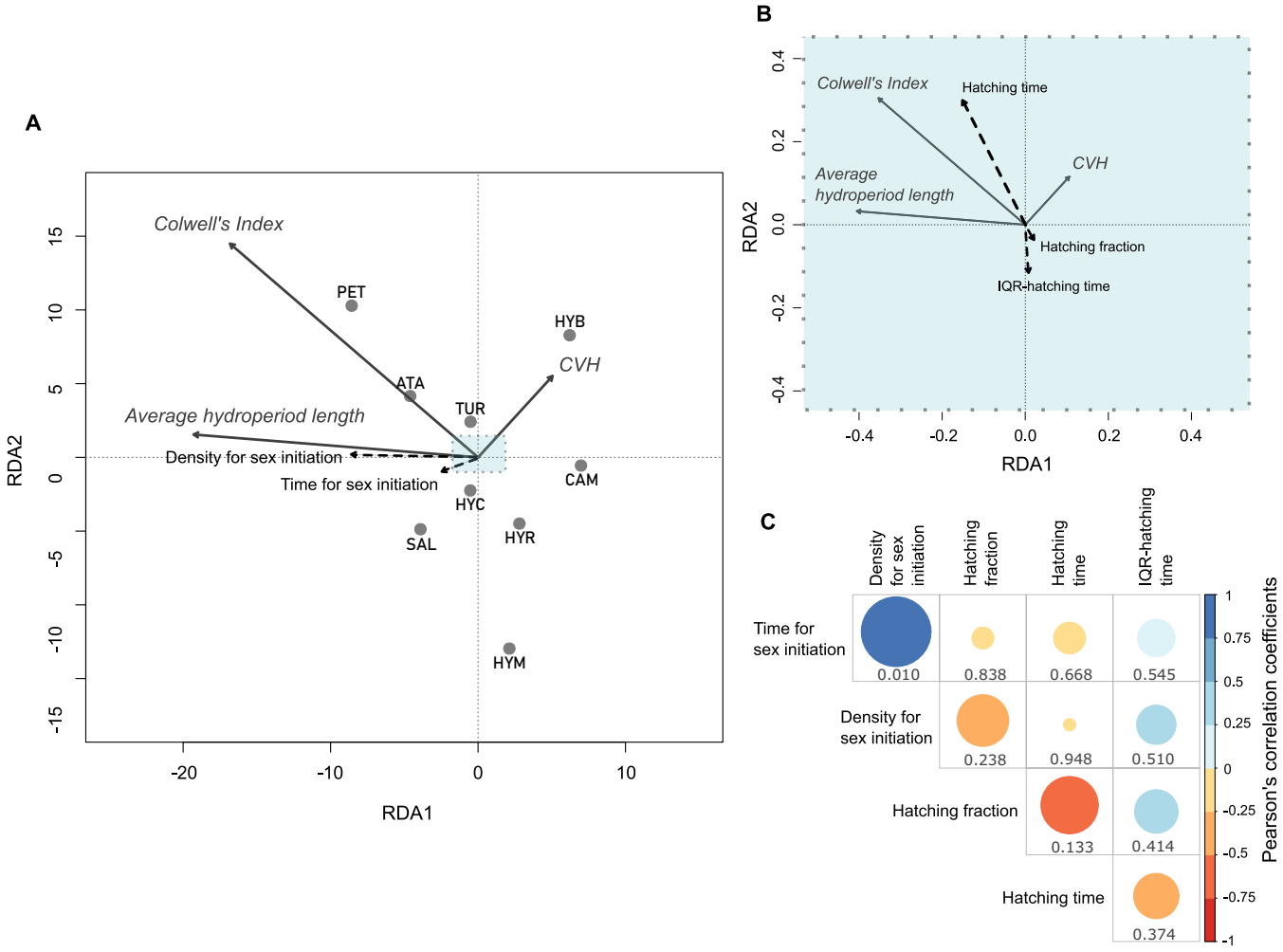

FIGURE 4 | (A) Ordination of the studied populations (dots) in the first two axes of the RDA on diapause-related traits (response variables) and environmental parameters (explanatory variables). RDA1 and RDA2 account for 59.5% and 0.8% of the constrained variance of data, respectively. Solid arrows represent environmental parameters, and dashed arrows represent the diapause-related traits (only two showed due to lack of resolution). (B) Zoom-in of the centre of the ordination plot showing the rest of the diapause-related traits (arrows as in A). (C) Correlation plot for pairwise comparison for the studied diapause-related traits. Circle area is proportional to the absolute value of the correlation coefficient. Corresponding p values are shown below each circle.

correlation found between these two metrics indicates that they account for different aspects of the environmental predictability, as might be perceived by rotifers; so that our a priori assumption of a two-fold concept seems to be justified.

We found signatures of temporal variation in our predictability metrics (i.e., non-stationary patterns), which raises the question of the time scale over which evolutionary processes shape the adaptive response of rotifers. Consistent with their large population size, short generation time and occurrence of recombination, it has been found that fast adaptive evolution of laboratory rotifer populations for diapause-related traits closely associated with fitness (e.g., Fussmann et al. 2003; Becks and Agrawal 2010, 2012; Smith and Snell 2012; Declerck and Papakostas 2016; Tarazona et al. 2017; White et al. 2022). These findings suggest the usefulness of using short periods to relate rotifer life-history traits and environmental parameters. Therefore, instead of using the entire dataset of available years as in Franch-Gras, García-Roger, Serra, and Carmona (2017); Franch-Gras, García-Roger, Franch, et al. (2017), we assumed that the relevant environment was in the decade preceding the sampling of rotifer populations. However, two caveats exist for using too short periods. First, unlike laboratory ones, natural populations are subject to multiple evolutionary forces that can slow down evolutionary processes, especially in fluctuating environments (Nadeau et al. 2017). Moreover, in natural populations heritability is expected to be lower than in laboratory ones, because controlled conditions decrease environmental variance. Second, using short periods results in reducing sample size and decreases confidence. This shortcoming is particularly expected in parameters based on variation, such as those for estimating predictability. Hence, given the conjectural character of this assumption, we examined whether the relationships between the focal life-history traits and the environmental parameters differed when the latter were estimated for periods shorter than a decade prior to rotifer sampling. We did not observe noticeable differences among the tested timespans (i.e., the previous 5, 7 and 10 years).

Given the crucial role of the survival of rotifer populations, diapause-related traits are thought to be key fitness components (Radzikowski and Beisner 2025; García-Roger et al. 2019). Here, by including hatching time and its variation, we enlarged the number of traits examined beyond those in previous studies, with a focus on traits related to diapause exit, in order to get a more complete picture of adaptation to environmental unpredictability. Our results identified variation among populations for nearly all traits, except for hatching time, which showed only a marginal response. Additionally, population variation in diapause-related traits not related to our unpredictability metrics suggests unmeasured selective factors at work. Furthermore, the high within-population genetic variation observed in our study also suggests potential for selection to act and for multiple genotypic strategies to coexist under fluctuating conditions.

In agreement with predictions from bet-hedging theory, results from our trait-by-trait analysis strongly suggest that unpredictable environmental variation promotes an early production of diapausing eggs in rotifer populations, since both the timing and density for sex initiation are negatively affected by the variation of hydroperiod length (CVH; hypothesis (b) in Section 1). This early investment in sexual reproduction is considered a conservative bet-hedging strategy for coping with unpredictability. If the end of the growing season is uncertain, investment in sexual reproduction as early as possible helps to avoid the risk of an unexpectedly short growing season, which could otherwise result in the complete reproductive failure of a rotifer genotype. Our result is in line with the conclusion drawn by Franch-Gras, García-Roger, Serra, and Carmona (2017), who also reported a relationship between predictability and early diapausing egg production. However, while their study assessed predictability only through interannual variation in the monthly distribution of hydroperiods (i.e., Colwell's index), our two-fold approach—incorporating also the variation in hydroperiod length (i.e., CVH)—revealed that the variation in the duration of hydroperiods is the factor involved in shaping reproductive strategies. Indeed, Tarazona et al. (2017) also observed rapid evolution toward an earlier timing of sexual reproduction in experimental rotifer populations subjected to a laboratory regime mimicking unpredictability in hydroperiod length. Worth noting, this strategy related to the production of diapausing eggs does not exclude a priori the existence of bet-hedging strategies for the exit from diapause. While not a direct test of bet hedging (*sensu* Simons 2011), we found some evidence for the latter, since rotifer genotypes from populations experiencing greater unpredictability exhibited greater variation in hatching time (i.e., higher values of IQR-hatching time were associated to lower values of Colwell's index; hypothesis (d) in Section 1). However, this evidence being weak, our results suggest that adaptation to unpredictability occurs mainly via the timing of diapausing egg production. Contrary to our expectations, hatching time did not increase with hydroperiod length (hypothesis (c) in Section 1).

The lack of evidence for some expected patterns in our results may be attributable to several reasons, beyond low statistical power in cases where the effect, although not statistically significant, aligns with the predicted direction (hypothesis (e) in Section 1). Certainly, bet hedging in a single trait might be enough to cope with environmental uncertainty, supporting the evolutionary hypothesis that at least one trait related to diapause should exhibit bet hedging. Additionally, given that bet hedging incurs costs, traits might be subject to trade-offs. Conversely, bet hedging might be scattered across several traits, resulting in a weak—difficult to detect—individual effect of each trait, but producing a sufficiently strong joint effect. On the other hand, our approach considered two types of unpredictability related to hydroperiod, and each one seems to be associated with different diapause-related traits. The inconclusive findings regarding the individual examination of potential bet-hedging traits may be due to the existence of syndromes (i.e., trade-offs and/or co-expression of two or more traits; Agrawal 2020) concerning the exit from diapause. This possibility was further supported by the ordination resulting from RDA, which suggested a negative relationship between hatching time on one side, and the fraction of hatching eggs and variation in hatching time on the other side (i.e., the other two traits associated to the exit of diapause).

Our understanding of strategies associated with the exit of diapause in rotifers remains less developed than that of diapause entry. To provide a conceptual framework that accommodates the hatching patterns observed in our results and fuel future research, we propose a conceptual model involving the three traits related to diapause exit: the hatching fraction, the variation in hatching

time relative to the growing season onset and the mean hatching time. For simplicity, each trait is represented at two levels—low or high hatching fraction, synchronous or asynchronous hatching, and early or delayed hatching time—resulting in eight possible trait combinations (Figure 5). We qualitatively relate these combinations to four ecological scenarios defined by growing season length (short or long) and predictability (low or high). Our aim is to present a general, yet realistic, qualitative framework that allows the formulation of hypotheses about the optimal trait combinations under simplified ecological scenarios. Although the number of trait combinations exceeds the number of scenarios, this mismatch may reflect alternative strategies suited to similar environmental conditions and may point to additional, unexplored components of environmental predictability.

The two strategies illustrated in Figure 5A involve early hatching and a low hatching fraction at the start of the growing season, with hatching occurring either synchronously (non-shaded curve) or spread out over time (shaded curve). Both strategies seem well-suited for environments with short and unpredictable hydroperiod length. Low hatching fraction protects against the risk of an unexpected end of the growing season, whereas spreading hatching could evolve if there were uncertainty about the onset of the growing season because of the likelihood of false starts. These are premature onsets of suitable conditions that do not persist long enough to allow survival and reproduction but may anticipate with some probability the actual advent of the season (Vanoverbeke and De Meester 2009; Kivelä et al. 2013; Ten Brink et al. 2020). Our results showed that the combination of some degree of asynchrony and low hatching fractions (as illustrated by the shaded curve in Figure 5A) is the commonest in our study system. This suggests that rotifer genotypes in most of these populations may experience unpredictability at both the start and the end—perhaps too premature—of the growing season. The early hatching generally observed in our study system is likely due to both habitat ephemerality and competitive pressure among clones to maximise exploitation of the growing season. The latter explanation is particularly compelling, as we did not find a relationship between hatching time and hydroperiod length, contrary to our original hypothesis.

In Figure 5B, one of the strategies (non-shaded curve) assumes early, massive and synchronous hatching. This strategy

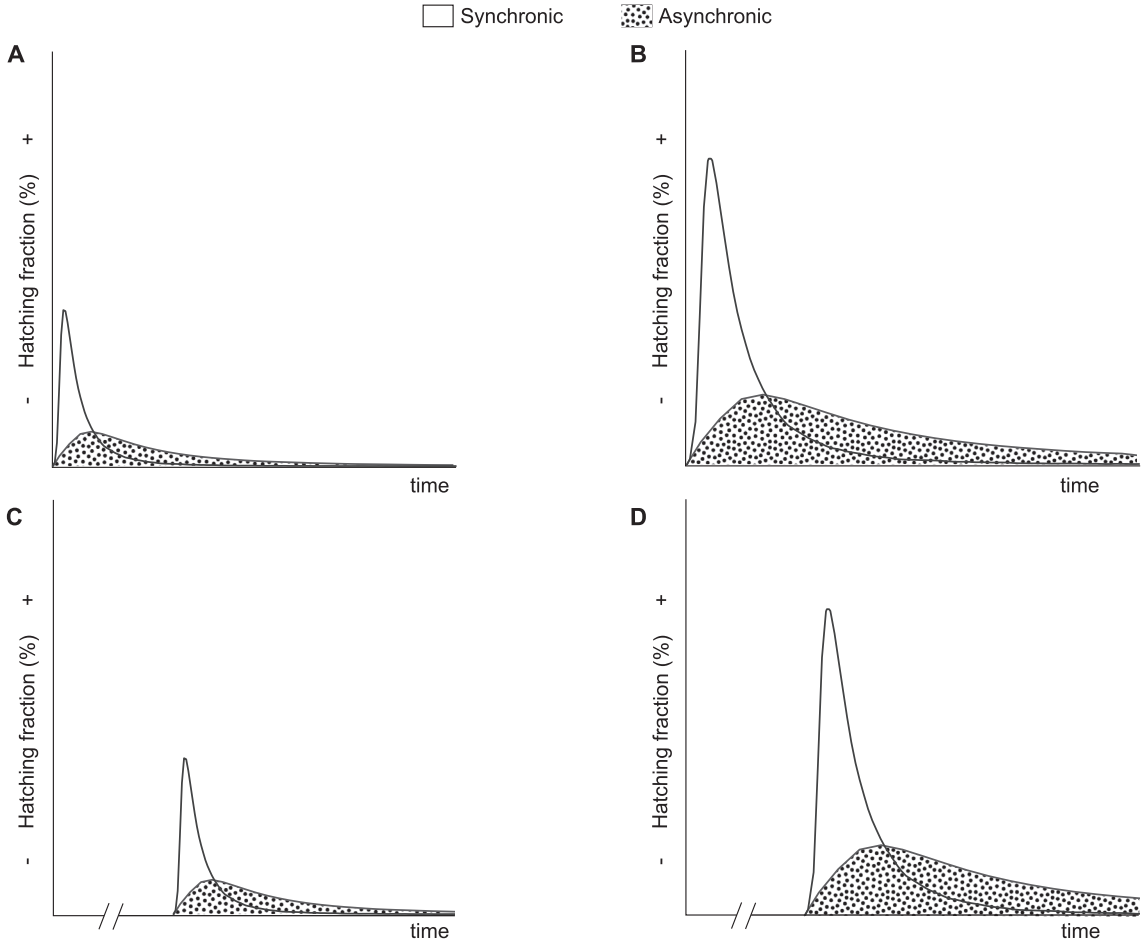

FIGURE 5 | Combinations of traits associated with exit from rotifer diapause, each with two levels: Early (top row) or delayed (bottom row) hatching time, low or high hatching fraction (according to ordinate axis) and synchronous or asynchronous (according to legend). (A) Early exit from diapause with low hatching fraction, synchronous or asynchronous. (B) Early exit from diapause with high hatching fraction, synchronous or asynchronous. (C) Delayed exit from diapause with low hatching fraction, synchronous or asynchronous. (D) Delayed exit from diapause with high hatching fraction, synchronous or asynchronous. A log-normal distribution of diapausing egg hatching over time was assumed for representation, and more or less asynchronous hatching does not correlate with mean hatching time.

is expected in a scenario where the length of the growing season is highly predictable but could prove fatal if any eventuality occurs. We certainly did not observe this strategy in any of the populations studied, suggesting that all are subject to some kind of environmental uncertainty. The shaded curve in Figure 5B represents a strategy characterised by a high total hatching fraction that is spread throughout the growing season. It would work in a scenario we think is more likely to occur than the former in our study system, where rotifer genotypes adopting this strategy would save a fraction of diapausing eggs, reducing the risk posed by either a randomly occurring very short growing season or the probability of a false start. Interestingly, HYM population fits in this pattern, exhibiting an early and dispersed hatching pattern and achieving a total hatching fraction higher than the other populations studied. Indeed, our results showed a negative relationship between hatching time and its variation, supporting the idea that if hatching occurs early in the growing season, asynchrony in hatching may protect against uncertainty at the onset of the growing season. Note that as a result of this type of strategy, the realised hatching pattern for an unexpectedly short growing season would resemble that of a low hatching fraction (Figure 5A non-shaded curve). This resemblance between strategies is particularly interesting, as we foresee that the expression of some of these traits may represent alternative adaptive responses to the same type of risk. Noteworthy, our results showed an overall positive relationship between hatching fraction and IQR-hatching time, evidencing a trade-off between the two traits. This means that spreading hatching within or between growing seasons could each address the unpredictability of the onset of the growing season. If this is the case, investing in only one of the two traits may be sufficient to respond to the challenge, thereby reducing the necessity for the full suite of bet-hedging traits related to the exit from diapause. But even more, intermediate hatching fractions may provide protection against unpredictability at both the onset and the end of the growing season (Ten Brink et al. 2020). This finding offers empirical support for the hypothesis derived from the general theory of bet hedging, which predicts that once a genotype has adopted a bet-hedging strategy and reduced variance in fitness, no additional risk-minimising strategy is selective target (Childs et al. 2010; Starrfelt and Kokko 2012). Notwithstanding, fully validating this requires the detailed characterisation of the two sources of unpredictability, a task for future research.

Strategies depicted in Figure 5C,D are characterised by delayed hatching times. As stated above, we did not find a statistically significant relationship between hatching time and hydroperiod length; delaying hatching does not appear to be adaptive per se. Thus, these trait combinations seem unlikely in our study system. Delayed hatching, because it is linked to asynchronous hatching (shaded curves in Figure 5C,D), might still be compatible with adaptation to risky environmental conditions at the onset of the growing season, a feature not considered in our model. Note that a property of the distribution assumed in our conceptual model is that an increase in the variance of the trait leads also to an increase in its mean. Thus, some delay in hatching times would always be observed as a consequence of adopting a strategy of spreading hatching throughout the growing season (see also shaded curves in Figure 5A,B).

Besides hydrological regime factors—namely, predictability and length of hydroperiod—, which act as the selective factors in our conceptual model—other environmental conditions could also help explaining the combinations of traits related to the exit of diapause in our conceptual model. This implies that the inverse inference—from traits to hydrological regime—might be problematic. As an instance, one putative factor not considered in our model is sediment adversity, which may favour higher hatching fractions (García-Roger et al. 2006, 2014). If the risk of not surviving in the sediment is high (e.g., due to predation, decay or burial in deep layers), high hatching fractions would be expected. In the face of risks in both the water column and the sediment, dispersing hatching across the growing season as in 5B (shaded curve) may be adaptive. Alternatively, frequency-dependent selection might be at work, causing departures in the predictions of our model (Serra et al. 2005). On the other hand, factors that were assumed to be unpredictable in our model might actually be predictable. If this would be the case, some other combination of diapause-related traits could arise as plastic responses to specific environmental cues that reliably inform about habitat suitability. If so, a delay in the recorded timing of diapausing egg hatching could be an artefact. An apparent, false delay, for instance, would occur if in our case the hydroperiod onset is not providing cues about the suitability of the growing season to start.

In summary, our data supported several of the originally proposed hypotheses, particularly those related to environmental unpredictability. Hypotheses that were not supported either showed statistically non-significant relationships (e.g., hypotheses (c) in Section 1) or exhibited non-significant trends that nonetheless warrant further investigation (hypothesis (e) in Section 1). Globally, our results showed that rotifers primarily respond to environmental uncertainty by adopting risk-averse strategies that conservatively ensure the earliest possible production of diapausing eggs, preparing for a potential unexpected end to the growing season. However, the observed substantial variation in the traits associated with the timing of diapausing egg hatching across the studied populations, along with the trait correlations described here, suggests that rotifer diapausing egg hatching patterns may be influenced also by the unpredictability at timescales lower than the length of the growing season. This study raises the possibility that hatching patterns do not evolve in concert. Instead, certain traits appear to respond to unpredictability associated with growing season length, while others respond to unpredictability within the growing season (e.g., the likelihood of false starts of the growing season). Clarifying whether these traits are responding through a form of diversifying bet hedging will require a thorough characterisation of rotifer habitats concerning the latter source of unpredictability, an elusive question not yet empirically addressed. Additionally, hatching patterns could be shaped by the combined effects of bet hedging and phenotypic plasticity. Diapausing eggs may respond to reliable environmental cues signalling the true onset of the growing season, or even if the growing season is appropriate, which could optimise reproductive timing, as proposed in predictive seed germination studies (Cohen 1967; Venable and Lawlor 1980; Simons 2014). Identifying which environmental cues could reliably inform rotifer eggs warrants experimental investigation. More broadly, hatching strategies might result from the combination of bet hedging and phenotypic

plasticity, fine-tuned to the levels of uncertainty and predictability perceived by rotifers (Donaldson-Matasci et al. 2013; Gremer et al. 2016). This paves the way for a promising yet underexplored area for future research.

Author Contributions

Conceptualization: Carlota Solano-Udina, Eduardo M. García-Roger, María José Carmona and Manuel Serra. Developing methods and conducting the research: Carlota Solano-Udina, Eduardo M. García-Roger, and María José Carmona. Data analysis: Carlota Solano-Udina, Manuel Serra and Eduardo M. García-Roger. Data interpretation: Carlota Solano-Udina, Eduardo M. García-Roger, María José Carmona and Manuel Serra. Preparation of figures and tables: Carlota Solano-Udina. Writing: Carlota Solano-Udina, Eduardo M. García-Roger, María José Carmona and Manuel Serra. Supervision: Eduardo M. García-Roger and María José Carmona.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.5vq2h.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Features of the studied ponds relevant in our analysis. Cloud check area (%) is the percentage of cloud-check area (an extension of 75-m width strip around each extent) relative to the corresponding extent. **Table S2:** Average hydroperiod length, coefficient of variation of hydroperiod length (CVH) and Colwell's index for predictability, calculated for each of the nine water bodies studied, presented in decade-based intervals (1984–1993, 1994–2003 and 2004–2013). **Table S3:** Statistical analysis for the effects of environmental parameters on diapause-related trait (i.e., average hydroperiod length, Colwell's index and the coefficient of variation in hydroperiod length, CVH) for the three addressed time spans (5, 7 and 10 years). Shaded cells are the results of unidirectional tests related with our expectations. RST, relationship statistically tested.