1 **Revealing population demographics with environmental RNA**

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3 Robert M. Hechler and Melania E. Cristescu

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5 **The analysis of environmental DNA (eDNA) and environmental RNA (eRNA)**

6 **released by organisms into their surrounding environment (water, soil, air), have emerged**

7 **as powerful tools for biodiversity monitoring. While eDNA has been widely adopted for the**

8 **non-invasive detection of species and characterization of community composition across the**

9 **tree of life, eRNA is in its infancy. Due to its functional nature, eRNA holds intriguing**

10 **potential for biodiversity monitoring opening new avenues of research beyond species**

11 **detection. For example, conspecifics that are almost genetically identical can exhibit**

12 **distinct transcriptomic differences depending on their life stage. In this issue of *Molecular***

13 ***Ecology Resources*, Parsley and Goldberg (2023) demonstrate, through a lab-validated field**

14 **study, that eRNA can be used to detect distinct life stages of amphibians. This study**

15 **elegantly demonstrates that eRNA can be used not only to detect invasive or endangered**

16 **species but also to reveal population demographic information important for conservation.**

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18 The greatest strength of eDNA lies in its ability to rapidly and non-invasively detect

19 species, but this may also be its Achilles' heel. Because within a population DNA remains

20 relatively constant, it is impossible to know the life stage, age, sex and phenotype of the

21 individual(s) contributing to the pool of DNA without visual identification (limitations of eDNA

22 for population genetics reviewed by Couton et al., 2023). On the other hand, the RNA profiles

23 can be distinct for different forms of conspecifics and Yates et al., (2021) speculated that these

24   differences might enable population demographic insights. Applications of eRNA are only now

25   emerging as it was long assumed that eRNA would degrade too rapidly to be recovered (but see

26   Cristescu, 2019). Recent empirical tests detected eRNA for up to 13 and 57 hours (Kagzi et al.,

27   2022; Wood et al., 2020). This rapid, but not too rapid, degradation of eRNA results in fewer

28   false-positive species detections and a more accurate representation of the 'living' community

29   members (Littlefair et al., 2022). Nevertheless, it remained unclear to what extent the eRNA

30   could overcome the limitations of eDNA and provide insights into the demographics of natural

31   populations.

32

33   Parsley and Goldberg (2023) performed the first eRNA based test for population

34   demographics for a pair of amphibians, the American bullfrog (*Lithobates catesbeianus*) and

35   tiger salamander (*Ambystoma mavortium*). They developed assays for specific larval life stages

36   and performed a laboratory validation study where organisms were separated not only by species

37   but also by life stage (adult versus larval). Their RT-qPCR from eRNA confirmed high

38   specificity of these assays to bullfrog tadpoles (90%) and salamander larvae (88.4%), which

39   mirrored the detection rate from skin samples used as positive control. Parsley and Goldberg

40   (2023) then tested the life stage specific eRNA assays in the field using three separate natural

41   systems, the American bullfrog and two closely related salamanders. In the presence of target

42   larval stages, they found high RT-qPCR eRNA detections in bullfrogs (74.1%) and long toed-

43   salamanders (70.8%) and a slightly decreased detection for the California tiger salamander

44   (48.5%).

45

46   Gaining insights beyond species detection via eRNA is a great advancement for non-

47   invasive biodiversity monitoring. Population demographic information is very important for

48   conservation and management as the survival and response of individuals to climate change may

49   depend on their life stage (Crozier et al., 2008) and sex (Hinch et al., 2021). Detecting larval

50   stages in nature could aid conservation efforts by identifying breeding locations and

51   distinguishing between breeding populations and transitory adults (Parsley and Goldberg, 2023).

52   In addition to life stage, eRNA could potentially reveal other population-level information that is

53   reflected in an organism's transcriptome (Figure 1). Yates et al., (2021) speculated that these

54   transcriptional differences may be reflected in eRNA, which would facilitate the discrimination

55   of different forms of conspecifics within a population. Indeed, emerging evidence suggests that

56   extra-organismal RNA extracted from the environment is representative of the organismal

57   transcriptional signal, as demonstrated by the targeted PCR life stage results from Parsley and

58   Goldberg (2023). Furthermore, analysis of RNA-sequencing reads originating from eRNA

59   revealed the gene expression stress response of progenitor *Daphnia* exposed to an experimental

60   heatwave (Hechler et al., 2023) and Japanese medaka fish exposed to sublethal pyrene levels

61   (Hiki et al., 2023). This emerging evidence suggests that eRNA incorporates 'true' signals

62   experienced by organisms and provides a promising outlook for potentially gaining additional

63   population demographic information (e.g. phenotypes, sexes and ages) as well as the

64   transcriptomic responses experience by populations under stress.

65

66   Although the potential of eRNA has been met with great enthusiasm by the eDNA

67   community, as with any other emerging tool, there is also need for vigilance, understanding and

68   overcoming potential problems and limitations. Fundamental questions about the ecology of

69   eRNA must first be answered as we currently do not have a clear picture about the production,

70   state, transport and fate of eRNA in natural systems (see outstanding questions posed by

71   Cristescu 2019 and Yates et al. 2021). This lack of information presents uncertainty for

72   determining optimal applications of eRNA and how to interpret results. For example, from initial

73   studies it seems that eRNA might be best applied to highly dense systems and might not be

74   always optimally suited for low-density/rare species. This is supported by Parsley and Goldberg

75   (2023) experiencing some false-negative eRNA detections in natural ponds where larvae were

76   visually confirmed to be present but in relatively low densities. We believe that continued

77   technological advancements will bring eRNA to the non-invasive biodiversity monitoring

78   toolbox.

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80   Monitoring biodiversity is critical in this period of environmental change and eRNA

81   holds great potential by providing more detailed insights into population demographics that are

82   important for conservation and management purposes. Parsley and Goldberg (2023) make a

83   pioneering contribution to the field as they clearly demonstrate that eRNA can be used to detect

84   specific life stages. We expect that their findings will provide a foundation for future research to

85   build upon and investigate whether eRNA can reveal additional population-level information

86   such as the proportion of different life stages, sexes, ages and phenotypes present in an

87   environment.

Environmental RNA: Potential Applications
- detect and partition the proportion of different life stages, sexes and phenotypes in populations
- assess health of populations
- detect metabolically active individuals
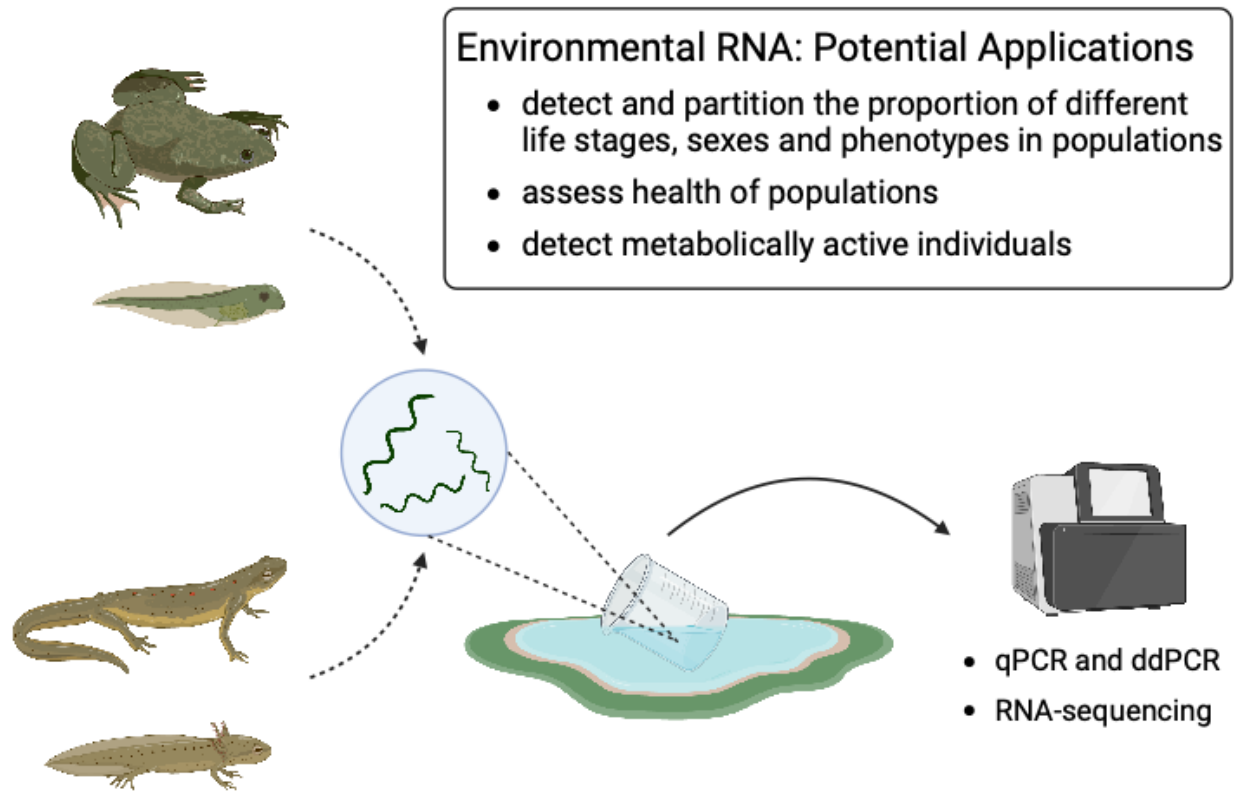
- qPCR and ddPCR
- RNA-sequencing

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89   **Figure 1.** Conceptual figure highlighting potential applications of environmental RNA for
90   population-level information as individuals from different species and life stages contribute to
91   the pool of environmental RNA. Figure created with BioRender.com.

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